Nitric oxide the gatekeeper of endothelial vasomotor control

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1. ABSTRACT

The endothelium can elicit relaxations and contractions of the underlying smooth muscle cells. It does so by releasing vasodilator (EDRF) and vasoconstrictor (EDCF) mediators. Among the diffusible endothelial factors nitric oxide (NO) plays a key role, particularly in large blood vessels. This chapter briefly reviews the interactions between NO and the other vasomotor signals released by the endothelial cells.

INTRODUCTION

Following the discovery of the endothelium-dependent character of the vasodilator responses to acetylcholine (1,2), it became obvious that such endothelium-dependency could not be explained by a single mechanism. Thus, we concluded in 1982 (3) that there were at least three pathways contributing to it, a proposal that withstood the test of time. Indeed, endothelium-dependent relaxations can be due to endothelium-derived relaxing factor (EDRF or nitric oxide (NO)), prostacyclin and/or endothelium-dependent hyperpolarization (EDHF-mediated relaxations) (see 4-9). Furthermore, when comparing the endothelium-dependent responsiveness of arteries and veins (10) we soon were confronted with the fact that endothelial cells not only release relaxing factors, but also can initiate endothelium-dependent contractions of the underlying vascular smooth muscle cells. Over the years, we have identified superoxide anions (11), thromboxane A_2 (12), endoperoxides (13) and prostacyclin (14) as cyclooxygenase-derived, endothelium-derived contracting factors (EDCF) (see 5, 15). Others discovered and identified the powerful vasoconstrictor peptide endothelin-1 (ET) that can be produced by endothelial cells (16,17).

As one became more and more confronted with the complexity of the endothelial control of local vasomotor tone, the concept emerged that several endothelium-derived vasoactive factors are produced concomitantly and that the resulting change in vascular diameter is not simply the addition of effects, but that one endothelial factor can influence the release or the bioactivity of the others that of the other (18-20). This brief review focuses on the central role of NO as a modulator of the release and/or the action of the other endothelium-derived vasoactive mediators.

3. NO AND ENDOTHELIUM-DEPENDENT HYPERPOLARIZATIONS

3.1. EDHF-mediated, endothelium-dependent relaxations

Hyperpolarization of the smooth muscle cells is a powerful way to produce its relaxation. It decreases not only Ca^{2+} influx by reducing the open probability of voltage-dependent Ca^{2+} channels (Ca_V) but also the release of Ca^{2+} from intracellular stores by decreasing the turnover of intracellular phosphatidylinositol and the Ca_V-dependent activation of the sarcoplasmic reticulum (8,21,22).

Numerous endothelium-derived factors, including not only NO itself but also carbon monoxide, reactive oxygen species, peptides and metabolites of arachidonic acid (derived from cyclooxygenases, lipoxygenases and cytochrome P450 monooxygenases), can hyperpolarize the underlying vascular smooth muscle cells by activating different families of K^+ channels. Another pathway, which does not involve the release of these factors but is associated with the hyperpolarization of both the endothelial and the vascular smooth muscle cells, contributes to endothelium-dependent relaxations. It involves an increase in endothelial intracellular calcium concentration, the opening of calcium-activated potassium channels of small and intermediate conductance (SK_{Ca} and IK_{Ca}) and the hyperpolarization of the endothelial cells. The hyperpolarization of the endothelial cell can be transmitted directly to the vascular smooth muscle by means of myoendothelial gap-junctions communication. Alternatively or concomitantly, the accumulation in the intercellular cleft of K^+ ions, released from the endothelial cells through the opening of SK_{Ca} and IK_{Ca}, hyperpolarizes the smooth muscle cells by activating inward rectifying potassium channels ($K_{IR}2.1$) and/or Na^+/K^+-ATPase (8, 9).

3.2. NO and EDHF-mediated responses
3.2.1. NO and potassium channels

NO activates BK_{Ca} (23, 24) via a PKG-dependent phosphorylation of the channel (25) or by a direct, cyclic-GMP-independent manner (26,27) as well as K_V (28,29) and K_{ATP} (30,31). Besides affecting K^+ conductances, NO also interacts with other ionic channels such as chloride and cationic channels and can thereby further influence the membrane potential of the smooth muscle cells (8).

NO can also activate smooth muscle K_{IR} (32), which are involved in EDHF-mediated responses, and activation of endothelial K_{IR} increases the release of NO (33,34). Although, NO and NO-donors activate SK_{Ca} and IK_{Ca} in non-vascular smooth muscle cells (35-38), little information is available on the potential role of NO in regulating the activity of these channels in endothelial cells. In the middle cerebral artery of the rat, NO enables endothelial SK_{Ca} activity in an indirect manner by preventing the synthesis of thromboxane A_2 and the subsequent activation of TP receptors (39,40). Long-term administration of nitroglycerine inhibits acetylcholine-induced endothelial hyperpolarization and the activation of IK_{Ca} by a mechanism involving the production of reactive oxygen species (41), as in the generation of nitrate

tolerance (42) and in the decrease of prostacyclin production (43). In murine mesenteric arteries, the gene expression of either SK_{Ca} or IK_{Ca} is unaffected by the deletion of the eNOS gene (44).

On the other hand, the activation of endothelial SK_{Ca} or IK_{Ca} is pivotal for an increase in NO synthesis (45-49, Figure 1). However, it remains uncertain whether or not endothelial K_{Ca}-dependent NO synthesis depends on the influx of extracellular calcium (48,50-54) or on other variables affecting the membrane potential (e.g. superoxide anion production (55) or L-arginine uptake (56)).

3.2.2. NO and connexins

The connexins (Cx) 37, 40 and 43 are the predominant isoforms of gap-junction proteins expressed in the vascular wall (57-59) and blockers of gap junctions abolish or partially inhibit EDHF-like responses in many arteries *in vitro* (57,60-66) and *in vivo* (67). Furthermore, in the rat mesenteric artery, antibodies directed against Cx40, when loaded selectively in the endothelial cells block EDHF-mediated responses (68).

NO can influence gap junction communications in cyclic GMP-dependent and independent ways or by direct nitrosylation of the connexins. NO is also involved in a positive and negative regulation of the expression of various connexins (69-73). However, EDHF-mediated responses of rabbit iliac arteries and of rat pial arterioles, the mechanism of which involves gap junctions composed of Cx37-Cx40 and Cx37-Cx-43, respectively, are not affected by either inhibition of NO-synthase or administration of NO donors (74,75).

In cultured human umbilical vein endothelial cells (HUVEC) stimulated with NO-donors and in transgenic mice overexpressing eNOS, the expression of Cx40 is increased (69,76), while eNOS inhibition does not affect the expression of Cx43 (77), and deletion of eNOS does not altered the vascular expression of Cx37, Cx40 and Cx43 (44,77). However, in L-NAME-induced hypertension, the aortic expression of Cx43 is reduced while, in other rat models, hypertension is associated with an increased Cx43 expression (78).

Mice with specific deletion of the vascular endothelial Cx43 gene are hypotensive and have an increased production of NO; the connection between the two events has not been established (79).

Therefore, although connexins are a target of NO, there is no evidence to date that NO influences EDHF-mediated responses by regulating gap-junctions communications (Figure 1).

3.2.3 NO and TRP channels

The major mechanism that sustains the opening of endothelial K_{Ca} channels, following stimulation, is the capacitive calcium entry elicited by the depletion of calcium stores (50,80) and which is associated with TRP channels activation (81-83). NO activates TRP channels of the TPRC and TPRV families by cysteine S-nitrosylation, and elicits calcium entry (84). The latter mechanism could
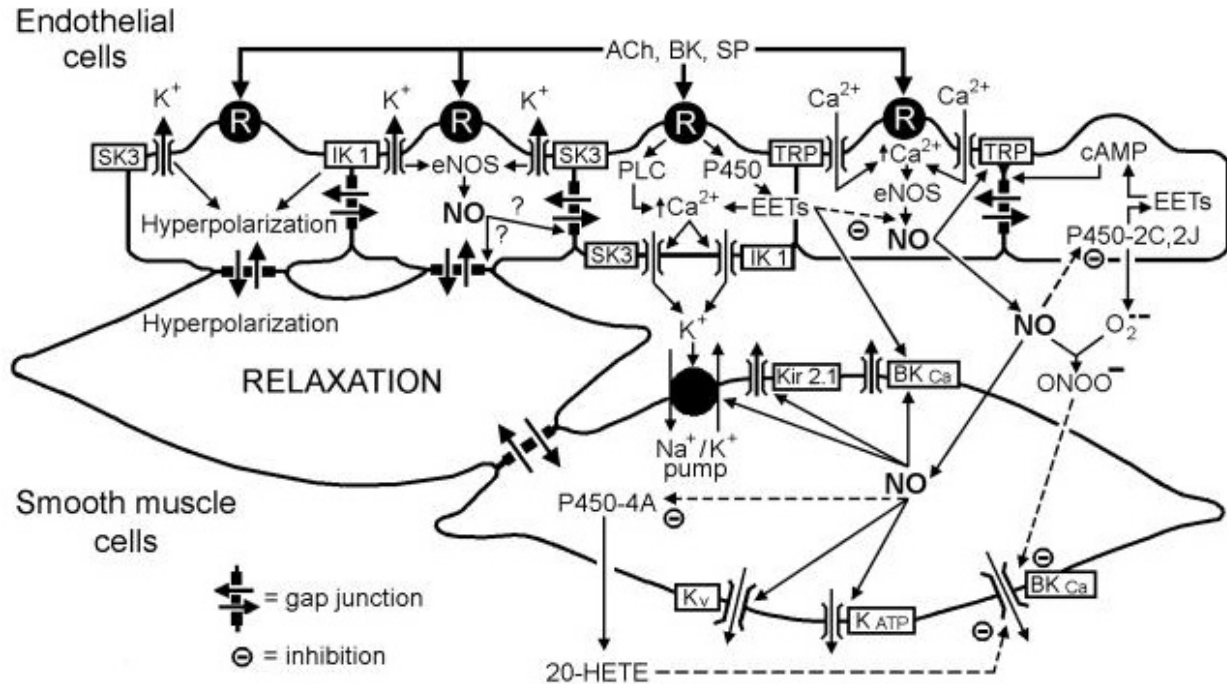
Figure 1. NO and endothelium-dependent hyperpolarizations. Interactions between nitric oxide (NO) and EDHF-mediated responses (associated with opening of small and intermediate conductance calcium-activated potassium channels) as well as between NO and cytochrome P450 metabolites (P450). R: receptor; ACh: acetylcholine; BK: bradykinin; SP: substance P; eNOS: endothelial nitric oxide synthase; EETs: epoxyeicosatrienoic acids; 20-HETE: 20- hydroxyeicosatetraenoic acid; O_2^{-}: superoxide anion; $ONOO^{-}$: peroxynitrite; PLC: phospholipase C; TRP: Transient Receptor Potential Channel; cAMP: cyclic-AMP; SK3: small conductance calcium-activated potassium channel formed by SK3 α-subunits; IK1: intermediate conductance calcium-activated potassium channel formed by IK1 α-subunits; Kir2.1: Inward rectifying potassium channel constituted of Kir2.1 α-subunits; K_V: voltage-gated potassium channels; BK_{Ca}: large conductance calcium-activated potassium channels; K_{ATP}: ATP-sensitive potassium channels.

constitute a positive and autocrine feedback loop by which NO enhances its own release and triggers EDHF-dependent vasodilatation (82,84; Figure 1).

3.2.4. NO and Na^+-K^+-ATPase

NO stimulates the Na^+-K^+-ATPase of vascular smooth muscle in a cyclic GMP-independent way, and the activation of the pump contributes to the relaxation (85-87). By contrast, it produces a cyclic GMP-dependent inhibition of the endothelial Na^+-K^+-ATPase (88,89). The inhibition of the endothelial Na^+-K^+-ATPase is associated with an increase in eNOS activity and NO production (90,91; Figure 1).

3.3. NO and lipoxygenases

In a limited number of blood vessels, lipoxygenase metabolites can be released by the endothelial cells and evoke relaxation of the vascular smooth muscle by activating K_{Ca} (8,92-94).

Lipoxygenases are non-heme iron dioxygenases and therefore do not contain the preferential target of NO. Nevertheless, the lipoxygenase-dependent formation of peroxynitrite, reduces and partially inhibits the enzyme activity (95,96). In hypercholesterolemic mice, the decreased contribution of NO in endothelium-dependent relaxations is compensated by metabolites of 12/15 lipoxygenase (97). On the other hand, in mice deficient in 12/15 lipoxygenase, the expression of eNOS and the production of NO are increased (98).

3.4. NO and cytochrome P450 monooxygenases

In endothelial cells, cytochrome P450 enzymes of the 2C or 2J families generate epoxyeicosatrienoic acids (EETs). EETs contribute to endothelium-dependent relaxations and hyperpolarizations in various blood vessels, including large and small coronary arteries (99,100), by activating BK_{Ca} (101,102). In vascular smooth muscle cells cytochrome P450 of the 4A and 4F families catalyze the ω-hydroxylation of arachidonic acid to produce hydroxyeicosatetraenoic acids (HETE). 20-HETE is a potent endogenous vasoconstrictor. The mechanisms underlying this vasoconstriction include inhibition of smooth muscle BK_{Ca} (103) as well as suppression of EDHF-mediated responses by inhibition of Na^+/K^+-ATPase (104, Figure 1).

NO binds to the heme moiety of cytochrome P450 monooxygenases and inhibits their activity. Additionally, NO decreases the expression of the

cytochromes P450 of the 2C family but increases that of the 4A family (103). In agreement with these observations, NO, derived from eNOS, iNOS or NO-donors, reduces endothelium-dependent hyperpolarizations that involve the generation of cytochrome P450 metabolites (105-107). In certain vascular beds of eNOS-genetically deficient mice, EETs compensate for the loss of NO production, for instance in response to flow (108; Figure 1).

In some peripheral vascular beds, the endogenous levels of 20-HETE are elevated and are responsible for an elevated intrinsic vascular tone. NO by inhibiting the enzymatic formation of 20-HETE indirectly activates BK_{Ca}, in a cyclic GMP-independent manner, and produces repolarization and relaxation (109,110; Figure 1). In eNOS knockout mice, the expression of cytochrome P450 A4 is not altered but the enhanced production of 20-HETE augments myogenic constriction (111).

The administration of EETs and the overexpression of cytochrome P450 2C enhance eNOS activity and increase the expression of the enzyme (112). Conversely, overexpression of cytochrome P450 4A produces hypertension, generates oxidative stress and reduces the bioavailability of NO (113).

In resistance arteries, the balance between EETs and 20-HETE, which have directly opposing effects, contributes to the control of vascular tone and NO plays a crucial role in regulating both pathways. NO, EETs and 20-HETE are not necessarily generated within the vascular wall. For instance, the neuron-astrocyte-endothelial signaling pathway is a major contributor in coupling blood flow to neuronal activity (114).

3.5. NO and heme oxygenase – carbon monoxide

The predominant biological source of CO is the degradation of heme by heme-oxygenase (115). An endothelial production of CO, contributing to endothelium-dependent relaxations in response to neurohumoral substances, is likely only in a limited number of arteries (8).

CO is a potent vasodilator in most, but not all, vascular beds. The mechanisms of CO-induced relaxation involve the stimulation of soluble guanylyl cyclase, the inhibition of cytochrome P450 and the activation of potassium channels. CO and CO-donors activate smooth muscle BK_{Ca}, K_{ATP} (116) and/or K_V (117). CO induces the cyclic-GMP-dependent activation of BK_{Ca} (100) but also directly increases the open probability of BK_{Ca} (118-122). Additionally, CO can also activate BK_{Ca} in a more indirect manner. CO, like NO, inhibits cytochrome P450 and suppresses the synthesis of 20-HETE, the endogenous vasoconstrictor and tonic inhibitor of BK_{Ca} (103,123,124).

The NO and CO systems further interact in a complex manner, to regulate vascular function. In many physiopathological situations the heme oxygenase-CO pathway compensates for the decreased bioavailability of NO (115,125). However, CO is a tonic inhibitor of NOS by binding to its prosthetic heme and can contribute to endothelial dysfunction (126-129). Both NO and peroxynitrite inhibit heme oxygenase activity by heme nitrosylation and oxidation of sulfhydryl groups, respectively (130-132).

3.6. NO and C-type natriuretic peptide

CNP, a member of the natriuretic peptide family, is produced in the central nervous system but has also been detected in peripheral endothelial cells and to a lesser extent in vascular smooth muscle cells. CNP evokes relaxations and hyperpolarizations of arterial and venous smooth muscle cells via a cyclic GMP-dependent activation of BK_{Ca} and or K_{ATP} (8, 133-136). Additionally, in the rat mesenteric artery, endothelium-derived CNP could activate NPR-C receptor subtype present in smooth muscle. Hyperpolarization of the smooth muscle cells could be evoked via the cyclic-GMP-independent activation of a G-protein regulated inward-rectifier K^+ channel (GIRK; 137,138). In other arteries, which also exhibit EDHF-mediated responses, the involvement of CNP has not been verified so far (136,139-141).

CNP in general causes endothelium-independent relaxations (133), although in some blood vessels, the peptide causes the release of endothelium-derived NO (142). Furthermore, CNP induces a post-transductional decrease in the level of the β subunit of soluble guanylate cyclase and reduces its activity (143).

NO produces a cyclic-GMP-dependent inhibition of the particulate guanylate cyclase associated-receptors, NPR-A and NPR-B (144,145) and inhibitors of NO-synthases potentiate the vasodilator and the hyperpolarizing responses to natriuretic peptides (136,145).

3.7. NO and hydrogen peroxide

Hydrogen peroxide (H_2O_2) possesses dilator and constrictor properties. It can be involved in endothelium-dependent relaxations and hyperpolarizations in response to agonists and flow (146-151). H_2O_2 can activate BK_{Ca} by a direct action on the channel as well as by an effect on soluble guanylyl cyclase (152,153), K_{ATP} (154), K_V (155), and/or K_{IR} channels (156). However, H_2O_2 does not always hyperpolarize vascular smooth muscle cells (157) and can even be a potent inhibitor of BK_{Ca} (158). Furthermore, in many arteries EDHF-mediated responses cannot be attributed to the generation of H_2O_2 (8,159,160) and in human coronary arteries it directly inhibits cytochrome P450 epoxygenases and the release of endothelial-derived hyperpolarizing epoxyeicosatrienoic acids (161).

The interactions between NO and H_2O_2 are multiple. Under physiological conditions, eNOS itself and Cu, Zn superoxide dismutase (SOD) appear to be the major contributors for the production of H_2O_2, as an endothelium-derived hyperpolarizing substance (149,162-164). Under pathological conditions H_2O_2 can compensate for the decreased production of NO, especially when the eNOS cofactor, tetrahydrobiopterin (BH_4), is depleted (165). H_2O_2 can produce endothelium-dependent relaxations via NO release (166,167), can increase either the expression of eNOS, through transcriptional and post transcriptional

mechanisms (168), or the activity of eNOS by augmenting the expression of GTP-cyclohydrolase (169). NO increases the expression of extracellular SOD, preventing the formation of peroxynitrite and increasing the probability of generating H_2O_2 (170). Additionally, H_2O_2 can share the same target as NO, since this reactive oxygen species directly activates the soluble guanylyl cyclase (171).

However, H_2O_2 can also cause vascular dysfunction (172). In mice overexpressing catalase, the pressor response to vasoconstrictors is decreased when compared to the wild type animals (173,174) and in transgenic mice, overexpressing human catalase specifically in vascular tissues, the arterial blood pressure is lower than in the wild type control (175). Similarly, the endothelium-dependent relaxations in arteries from glutathione peroxidase deficient mice are impaired (176,177) while overexpressing this enzyme can compensate for the adverse effects of hyperhomocysteinemia on endothelial function (178). Therefore, endogenously produced H_2O_2 favors vascular dysfunction either by direct vasoconstrictor effects on resistance vessels and/or by producing endothelial dysfunction possibly by decreasing eNOS gene expression (179) or by inactivation of eNOS cofactors (180).

Additionally, NO regulates many of the enzymatic systems producing superoxide anions. For instance, it decreases the expression of two NAD(P)H oxidase subunits, Nox2/gp91(phox) and p47(phox) (181), and can decrease NAD(P)H activity directly by S-nitrosylation of p47(phox) (182) and indirectly via the induction of heme oxygenase-1 (183).

3.8. Conclusion

The endothelium-dependent control of vascular tone is essential for local vascular homeostasis. Hence, it comes as no surprise that compensatory pathways are put forward when the NO-bioavailability is reduced. For instance, in eNOS knockout mice, nNOS and cyclooxygenase derivatives partially compensates for the disruption of the endothelial isoform (184-189). Similarly, in various animal models as well as in the human, a compensatory role has been attributed to the NO-synthase- and cyclooxygenase-independent responses. This is the case for instance, in eNOS knockout mice (190,191) in double eNOS-COX1 knockout mice (192), in a murine model of hypercholesterolemia (193), in rat and murine models of diabetes (194,195), in rats with heart failure (196,197), in rat with L-NAME-induced hypertension (198), in Sprague-Dawley rats fed a high salt diet (199) and in hypertensive humans (200).

It has often been proposed that EDHF-like responses not only compensate for the absence of NO but that the latter exerts a tonic repression on the former (20,105). EDHF-like responses would be a backup mechanism unveiled under pathological conditions of decreased NO bioavailability. When cytochrome P450-dependent responses are involved, a mutual compensation of these and NO-dependent responses seems plausible (105,201-207). Indeed, NO can directly inhibit the

production of EETs by interacting with the heme moiety of the enzyme and can mask the effect of EETs by activating the same target in the smooth muscle cells, i.e. BK_{Ca}. Similarly the production of H_2O_2 by NO-synthase occurs mainly when the production of NO is jeopardized (149,165).

Nevertheless, this direct reciprocal interaction between NO and EDHF-mediated responses is far from systematically verified. In the murine perfused hindlimb, EDHF-mediated responses can compensate for the lack of NO in both wild type animals treated with a NOS inhibitor and eNOS knockout mice. Conversely, a NO-dependent contribution is observed in the wild type mice only when the EDHF-mediated responses are blocked (191). However, although prior blockade of either NO or EDHF-mediated response is required to unmask the contribution of either endothelium-dependent vasodilator mechanism, this does not indicate that the two systems are directly linked. Indeed, in wild type mice treated with a NOS inhibitor, the restoration of NO levels (by infusing an NO donor) does not affect the EDHF-mediated responses (208). Similarly, EDHF-mediated responses in rat cerebral arteries (209,210) or in the human forearm (211) are independent of NO. Furthermore, a pathway can be specifically affected without altering the other. For instance, in SHR and steptozotocin-treated rats, the EDHF-mediated responses are reduced markedly, while the NO-dependent component of the endothelium-dependent relaxation is not modified (212-214). In SHR, treatments with converting enzyme inhibitors or AT1 receptor blockers restore the altered EDHF-mediated responses without affecting the NO-dependent relaxations (215,216). In other models of hypertension, both the NO-component and the EDHF-mediated responses could be decreased (217,218) while in the L-NAME-treated guinea-pig, EDHF-mediated responses are unaffected (219).

Therefore, endothelium-dependent responses resistant to inhibitors of cyclooxygenases and NO synthases are not necessary a backup mechanism that gets into action only when NO bioavailability is decreased (8,187).

4. NO AND EDCF

4.1. Release of EDRF and EDCF

Agonists that elevate intracellular calcium concentration evoke the simultaneous release of both relaxing (EDRF) and contracting (EDCF) factors from the endothelium to control the tone of the underlying vascular smooth muscle. The mechanical consequence of the stimulation by any agonist relies on the net algebraic sum between the degrees of relaxation versus the degree of contraction. Nitric oxide is the principal EDRF in many arteries (220). Its synthesis involves the activity of endothelial nitric oxide synthase (eNOS), which is calcium-dependent (220,221). Nitric oxide diffuses to the smooth muscle and stimulates soluble guanylyl cyclase to produce cyclic guanosine monophosophate (cyclic GMP) causing relaxation (222). Endothelium-dependent contractions are mediated by products of cyclooxygenases (15, 19). The

raise in intracellular calcium stimulates phospholipase A_2, which frees arachidonic acid for the metabolism by the cyclooxygenase (223). The breakdown of fatty acid by the cyclooxygenase generates constrictor prostanoids (such as endoperoxides, prostacyclin and thromboxane A_2) and reactive oxygen species (11,13,14,224-229). These mediators are termed EDCF. They ultimately activate TP receptors of the smooth muscle to evoke contractions (225,230). Under normal conditions, the releases of EDRF and EDCF are balanced and a normal vascular tone is maintained. However, in aging and in a number of diseases such as hypertension, diabetes and atherosclerosis, the balance between EDRF and EDCF release becomes dysfunctional and the generation of EDCF is favored (15,229-234).

4.2. Acute inhibition of endothelium-dependent contractions by nitric oxide

Inhibitors of nitric oxide synthase (such as N^{ω}-nitro-L-arginine methyl ester; L-NAME or N^G-monomethyl L-arginine; L-NMMA) or scavengers of nitric oxide (oxyhemoglobin or carboxyl-PTIO), which interfere with the production and the transfer of nitric oxide, respectively, augmented endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat (SHR) (19). By contrast, methylene blue used as an inhibitor of soluble guanylyl cyclase did not affect endothelium-dependent contractions (19), suggesting that the inhibitory effect of nitric oxide on endothelium-dependent contraction is independent of this enzyme. From these observations, nitric oxide was hypothesized to inhibit EDCF-mediated contractions by chemical inactivation, rather than functionally counteracting each other. However, methylene blue, at the concentration commonly used to inhibit guanylyl cyclase (10^{-4} M) had little effect on the accumulation of cyclic GMP induced by sodium nitroprusside (235). By contrast, oxadiazolo (4, 3-a)quinoxalin-1-one (ODQ; at 10^{-5} M, a selective inhibitor of guanylate cyclase) produces nearly complete inhibition of the formation of the cyclic nucleotide under the same conditions (235). Furthermore, methylene blue is not only an ambiguous inhibitor of guanylyl cyclase, but it exerts many non-specific effects that are directly associated with endothelium-dependent contractions. In particular, methylene blue inhibits muscarinic receptors (236,237), including the M3 subtype which is specifically involved in the release of EDCF by acetylcholine (238). It also suppresses the metabolism of arachidonic acid and the synthesis of prostacyclin (239,240), postulated to be the main mediator accounting for the acetylcholine-induced endothelium-dependent contraction (14). Methylene blue also affects the contractile apparatus of smooth muscle as judged from the observations that it reduces contraction to potassium chloride and isoprostanes (241). Thus, the use of methylene blue to estimate the involvement of guanylyl cyclase in the inhibitory effect of nitric oxide on endothelium-dependent contraction is not at all convincing.

When specific guanylyl cyclase inhibitors (such as ODQ and NS-2028) became available, the involvement of this enzyme in the inhibitory effects caused by nitric oxide on endothelium-dependent contraction was re-

examined. Both ODQ and NS-2028 were as effective as inhibitors of nitric oxide synthase or nitric oxide scavengers in increasing endothelium-dependent contractions (241), illustrating that the activation of guanylyl cyclase is indeed involved. The combined administration of nitric oxide synthase inhibitors or nitric oxide scavengers with guanylyl cyclase inhibitors did not have an additive enhancing effect on endothelium-dependent contractions (241). These studies imply that the inhibition of endothelium-dependent contractions by nitric oxide is through functional antagonism rather than through the direct inactivation process as concluded with the use methylene blue. The acute functional antagonizing effect of nitric oxide on endothelium-dependent contractions justifies the addition of nitric oxide inhibitors, nitric oxide scavengers and/or guanylyl cyclase inhibitors to vascular preparations to unmask the occurrence of EDCF-mediated responses and therefore facilitate their study.

Endothelium-derived reactive oxygen species behave as an EDCF, at least in certain arteries, such as the basilar artery of the dog (11,242) and the renal artery of the rat (243) by causing direct contraction of the underlying smooth muscle. However, in other arteries of other species, it remains controversial whether reactive oxygen species are the EDCF themselves or act as facilitator of endothelium-dependent contractions (224,228,229,244-247). Whatever the case, the ability of reactive oxygen species to scavenge nitric oxide can result in impaired relaxation and a higher propensity to develop endothelium-dependent contractions (248-250).

4.3. Long-term modulation of endothelium-dependent contractions by nitric oxide

Aortic rings of SHR which are previously exposed to nitric oxide donor such as sodium nitroprusside or endothelium-dependent vasodilators such as acetylcholine that release NO have a reduced ability to produce EDCF-mediated contractions afterward (251). This suppressive role of nitric oxide on endothelium-dependent contraction is a time- and concentration-dependent process. Exposure to minimal active amount of sodium nitroprusside (as low as 10^{-9} M) or exposure to 10^{-4} M sodium nitroprusside for a mere five minutes significantly hampered the magnitude of the subsequent endothelium-dependent contractions (251). These studies illustrate that nitric oxide not only exert acute inhibitory effects on endothelium-dependent contractions but it can also negatively modulate their occurrence in a longer lasting manner. Pre-exposure to sodium nitroprusside inhibited the subsequent endothelium-dependent contractions evoked by both acetylcholine and the calcium ionophore A23187 (a receptor-independent agonist that opens pores in the cell membrane and permits the free entry of extracellular calcium in the endothelial cells following its concentration gradient (252)) (251). Such observation suggests that the nitric oxide donor inhibits EDCF-mediated response through an event that is downstream of the rise in calcium concentration. The changes are unlikely to be at the level of vascular smooth muscle, since U46199, a synthetic TP receptor agonist, evoked similar responses in rings with or without pre-exposure to the nitric oxide donor (251).
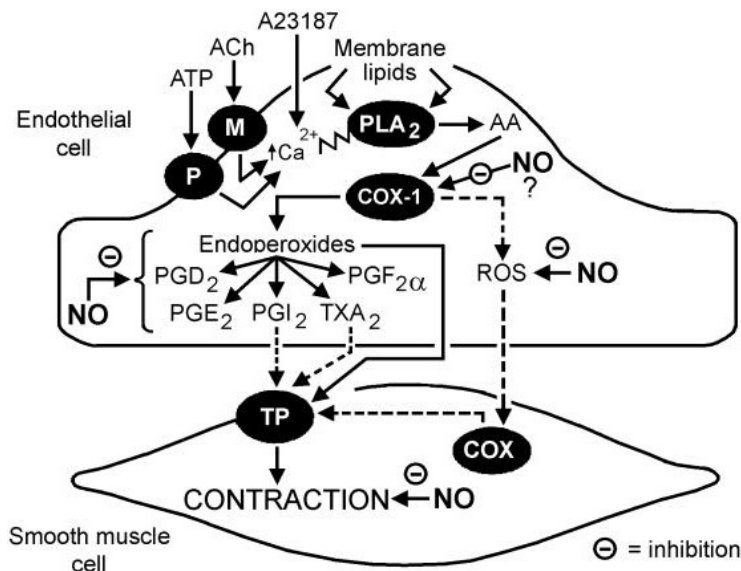
Figure 2. NO and EDCF. Nitric oxide not only functionally antagonizes EDCF-mediated contractions, but it can scavenge reactive oxygen species and decrease the bioavailability of EDCF, presumably at the site of the endothelial cells. AA = arachidonic acid; ACh = acetycholine; ADP = adenosine diphosphate; m = muscarinic receptors; P = purinergic receptors; PGD_2 = prostaglandin D_2; PGE_2 = prostaglandin E_2; $PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$; PGI_2 = prostacyclin; PGIS = prostacyclin synthase; PLA_2 = phospholipase A_2; ROS = reactive oxygen species; TXA_2 = thromboxane A_2; TXAS = thromboxane synthase.

Therefore, the changes exerted by pre-exposure to sodium nitroprusside most likely involve an alteration of EDCF bioavailability that takes place within the endothelial cells (Figure 2).

The inhibition of acetylcholine-mediated endothelium-dependent contractions caused by pre-exposure to sodium nitroprusside in SHR aortic preparations was partially restored by the the guanylyl cyclase inhibitor, ODQ, suggesting that the inhibitory process involves both a cyclic GMP-dependent and -independent effect (251). Controversially, ODQ did not prevent the inhibitory effect caused by pre-exposure to sodium nitroprusside on EDCF-mediated contractions mediated by the calcium ionophore A23187 (251). The endothelium-dependent contractions by acetylcholine and the calcium ionophore A23187 are not entirely identical in nature. Endoperoxides and prostacyclin are the main mediators accounting for the endothelium-dependent contractions mediated by acetylcholine (13,14). In contrast, endoperoxides and thromboxane A_2 are the main contributor to the EDCF-mediated contraction to A23187 (226). Nitric oxide may modulate each different prostanoid synthases differently. Pre-exposure to nitric oxide may selectively decrease the expressions and/or the activities of prostacyclin synthase and thromboxane synthase and perhaps only the inhibition of the former is cyclic GMP-dependent. Such hypothesis may explain the discrepancy in the restoration with ODQ in endothelium-dependent contractions evoked by acetylcholine and A23187. Nitric oxide can influence the enzymatic activity of cyclooxygenase by interacting with the iron-heme group which is needed as a co-factor for the enzyme (253,254). By direct binding, it can modulate the protein structure of

the enzyme (253,254). Such mechanisms may account for the cyclic GMP-independent inhibition of endothelium-dependent contractions.

4.4. Conclusion

Care must be taken to design an experimental protocol with minimal previous exposure to agonists which release nitric oxide during studies of endothelium-dependent contractions, or else EDCF-mediated response can be severely hampered. If the presence of the endothelium or the viability of an arterial ring needs to be tested, short exposures to a single dose of acetylcholine are recommended in order to avoid the attenuation of the endothelium-dependent contraction.

Nitric oxide and EDCF exert opposing effects on the vascular smooth muscle and thus behave as acute functional antagonists. Nitric oxide also negatively controls and modulates the occurrence of endothelium-dependent contractions in a longer lasting manner. Under conditions of reduced nitric oxide bioavailability, as caused by the endogenous release of nitric oxide inhibitors such as asymmetric dimethylarginine (255,256) or by the enhanced formation of superoxide anions which scavenge the relaxing factor (18), the brakes on the development of EDCF-mediated response and the overall opposing effect on the contraction are weakened and this leads to the emergence of endothelium-dependent contractions.

5. NO AND Endothelin-1

5.1. Release and action of endothelin-1

A turning point in the quest to identify EDCFs was the discovery that besides superoxide anions,
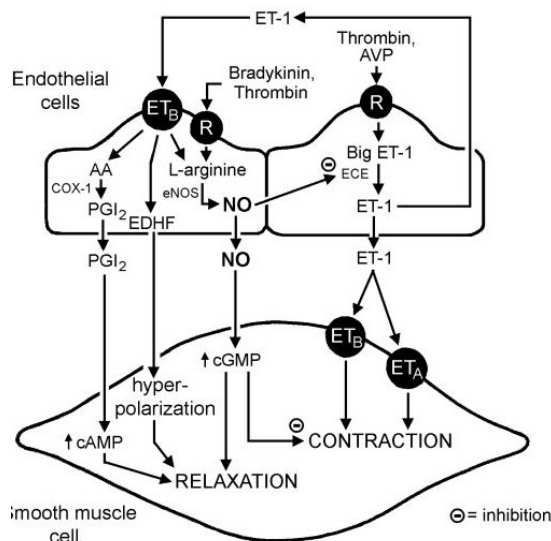
Figure 3. NO and endothelin. Release and actions of endothelin-1 (ET-1) in the vascular wall. AA : arachidonic acid ; AVP = arginine vasopressin ; cAMP = cyclic AMP ; cGMP = cyclic GMP ; COX= cyclooxygenases; ECE = endothelin converting enzyme ; ET_A and ET_B = endothelin-receptors ; NO = nitric oxide ; NOS = nitric oxide synthase ; PGI_2 = prostacyclin ; R = cell membrane receptor

endoperoxides, and thromboxane A_2 endothelial cells can produce potent vasoconstrictor peptides (16,257). These peptides were identified by Masaki and colleagues (17) and termed "endothelins" (ET), although it soon became obvious that cells other than the endothelium produce them, and that indeed their role in development and/or physiology may well lie with other cell types (258). Endothelin-1 was for several years the most potent vasoconstrictor substance known (e.g. 259-263).

After the discovery of the endothelins, the attempts to link it to the normal control of moment-to-moment changes in vascular tone did not yield very convincing data (e.g. 264). Likewise, a causal or early role in pathologies such as hypertension (e.g. 265,266) was not obvious. However, its presence in, and possibly its contribution to the terminal stages of vascular (and other) diseases became probable (e.g. 267-272). The availability of endothelin-antagonists for the use in humans has confirmed that endothelin-1 may progressively become more important with age (273,274) and in diseases such as pulmonary hypertension (e.g. 272,275,276). In the latter case the trophic, mitogenic effect of endothelin-1 must play a key role (277) besides the direct vasoconstrictor effect of the peptide (278). When the concept emerged that the production of the peptide is a sign of pathology and that under normal circumstances it plays little role in vascular homeostasis (261,279). The production of endothelin-1 once initiated not only progresses linearly with time (at least in cultured endothelial cells) but also can be up regulated by a number of factors believed to play a role in vascular disease (e.g. 280). Thus one suggested that under normal physiological conditions the production and/or the action of endothelins could not proceed unmatched (279).

5.2. Modulation by NO
In a number of blood vessels, nitric oxide synthase and endothelin-1 co-localize, implying interactions between the two mediators (e.g. 281-284). A key finding was the demonstration that stimulation of the production of NO inhibits the expression and the production of endothelin-1 (285,286), an observation that has been reproduced repeatedly and extended to other means of increasing the intracellular concentration of cyclic GMP (287-290). Likewise, when confronted with the extraordinary powerful and sustained vasoconstrictor potency of the peptide, it soon became obvious that the administration of exogenous nitric oxide, or its liberation from endothelial cells was a very good way to deal with it (e.g. 291,292;), as NO attenuates in a cyclic-GMP dependent way the activation by endothelin-1 of the signaling cascade leading to the contraction of vascular smooth muscle (293,294). This is more than another pharmacological action of NO. Indeed, if endothelin-1 reaches normal endothelial cells it activates receptors (of the ET_B subtype) on their cell membrane which are linked to the production of nitric oxide through pertussis-toxin sensitive G-proteins (295,294-306). Hence, any tendency to (over) produce the peptide in endothelial cells would be offset, under normal conditions, by the increased release of NO which automatically will reduce the generation of endothelin-1 and curtail its vasoconstrictor (and growth-stimulating) effects. This feedback inhibition of the production and action of endothelin probably is not limited to nitric oxide, as it also augments the release of prostacyclin (which by activating adenylyl cyclase further inhibits the production of the peptide) and EDHF (which contributes to the inhibition of its vasoconstrictor properties (307,308) (Figure 3).

The dual action of nitric oxide (and possibly of prostacyclin and EDHF) on the release and the action of endothelin provides a satisfactory explanation for the minor contribution of the peptide in local vasomotor conditions under normal circumstances. As the endothelium ages

and/or regenerates (and in particular loses the G_i-proteins mediated responses; 296,309-311), the buffering action on the production of, and the inhibition of the response to endothelin disappears (e.g. 312). Hence the peptide begins to contribute to vascular abnormality, a situation carried to the extreme in the terminal stages of cardiovascular disease (267,313-317). One of the most convincing demonstration of the unleashing of the production of endothelin-1 by the absence of nitric oxide are the repeated observations that the sustained increases in arterial blood pressure caused by inhibitors of nitric oxide synthase (originally attributed solely to a permanent state of vasodilatation maintained by the ongoing release of nitric oxide (6,318)), in fact are nearly prevented by antagonists of ET receptors, which are the primary effectors of the vasoconstrictor response to the peptide (288,319-333).

5.3. Conclusion

In the vascular wall, it seems reasonable to conclude that as long as enough nitric oxide (and possibly prostacyclin and EDHF) are produced by the endothelial cells endothelin contributes little to vascular homeostasis. Only when the endothelium loses its ability to generate EDRFs, the peptide can be generated in sufficient amounts to contribute to the symptoms of vascular disease.

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Abbreviations: BH_4: tetrahydrobiopterin; BK_{Ca}: calcium-activated potassium channels of big conductance; CNP: C-type natriuretic peptide; CO: carbon monoxide; COX: cyclooxygenase; Cx: connexins; EDCF: endothelium-derived contracting factor; EDHF: endothelium-derived hyperpolarizing factor; EDRF: endothelium-derived relaxing factor; EETs: epoxyeicosatrienoic acids; eNOS: endothelial nitric oxide synthase; ET: endothelin; GIRK: G-protein regulated inward-rectifier K^+ channels; GMP: guanosine monophosphate; H_2O_2: hydrgen peroxide; HETE: hydroxyeicosatetraenoic acids; HUVEC: human umbilical vein endothelial cells; IK_{Ca}: calcium-activated potassium channels of intermediate conductance; iNOS: inducible nitric oxide synthase; K_{ATP}: ATP-senstive potassium channels; K_{IR}: Inward rectifying potassium channels; K_V: voltaged-gated potassium channels; L-NAME: N^ω-nitro-L-arginine methyl ester; L-NMMA; N^G-monomethyl L-arginine; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; NPR: natriuretic peptide receptor; ODQ: oxadiazolo (4,3-a)quinoxaline-1-one; PKG: protein kinase G; SHR: spontaneously hypertensive rats; SK_{Ca}: calcium-activated potassium channels of small conductance; SOD: superoxide dismutase; TP: thromboxane-prostanoid receptor; TRP: transient receptor potential channels.

Key Words: Endothelium-derived hyperpolarizing factor, EDHF, Endothelium-Derived Contracting Factor, EDCF, endothelin-1, ET, Nitric Oxide, Connexins, TRP channels, Na^+-K^+-ATPase, Lipoxygenase, Cytochrome P450 Monoxygenases, Heme Oxygenase, Carbon Monoxide, C-Type Natriuretic Peptide, Hydrogen Peroxide

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