

Review

COX-mediated endothelium-dependent contractions: from the past to recent discoveries

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Endothelial cells release various substances to control the tone of the underlying vascular smooth muscle. Nitric oxide (NO) is the best defined endothelium-derived relaxing factor (EDRF). Endothelial cells can also increase vascular tone by releasing endothelium-derived contracting factors (EDCF). The over-production of EDCF contributes to the endothelial dysfunctions which accompanies various vascular diseases. The present review summarizes and discusses the mechanisms leading to the release of EDCFs derived from the metabolism of arachidonic acid. This release can be triggered by agonists such as acetylcholine, adenosine nucleotides or by stretch. All these stimuli are able to induce calcium influx into the endothelial cells, an effect which can be mimicked by calcium ionophores. The augmentation in intracellular calcium ion concentration initiates the release of EDCF. Downstream processes include activation of phospholipase A₂ (PLA₂), cyclooxygenases (COX) and the production of reactive oxygen species (ROS) and vasoconstrictor prostanoids (endoperoxides, prostacyclin, thromboxane A₂ and other prostaglandins) which subsequently diffuse to, and activate thromboxane-prostanoid (TP) receptors on the vascular smooth muscle cells leading to contraction.

Keywords: cyclooxygenase; EDCF; endothelium; gap junctions; phospholipase A₂; prostanoids; reactive oxygen species; TP-receptors

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Introduction

Following the first report by Furchgott and Zawadzki (1980)^[1] that in response to acetylcholine, endothelial cells release a vasodilator substance [endothelium-derived relaxing factor (EDRF)] later identified as nitric oxide (NO), a number of other inhibitory endothelial signals [endothelium-derived hyperpolarizing factors (EDHF)] have been shown to contribute to relaxations of the underlying vascular smooth muscle cells^[2–9]. In addition, it soon became apparent that under certain circumstances the endothelium can also produce diffusible substances [endothelium-derived contracting factors (EDCF)] which activate the contractile process in the underlying vascular smooth muscle cells^[10]. Besides receptors-mediated agonists such as thrombin, acetylcholine and adenosine nucleotides (ADP and ATP)^[11–13], stretch can also elicit endothelium-dependent contractions, at least in canine cerebral arteries^[14]. The early observation that such endothelium-dependent contractions could be prevented by inhibitors of cyclooxygenase suggested that down-stream products of this enzyme, *ie* prostanoids, were likely candidates as EDCF^[12, 15–18]. Although endothelial cells can produce vasoconstrictors including

endothelin-1 and angiotensin II, there is lack of convincing evidence showing a direct link between these substances and instantaneous changes in tension that can be attributed to the release of EDCF. Thus, the present article focuses on the mechanisms leading to the production of endothelial and cyclooxygenase-derived vasoconstrictors, and updates earlier reviews on this topic^[19–21].

Endothelial calcium concentration

An increase in intracellular calcium concentration in the endothelial cells is the triggering event leading to the release of EDCF. This conclusion is based on the following observations: (a) Activation of cell membrane receptors by agonists such as acetylcholine [activating endothelial M₃-muscarinic receptors^[22]], ADP and ATP [activating purinoceptors^[11, 23], which are known to induce the release of calcium from the sarcoplasmic reticulum^[24], initiate the production of EDCF; (b) Reduction in the extracellular calcium concentration decreases endothelium-dependent contractions^[25]; (c) Calcium ionophores such as A23187 elicit endothelium-dependent contractions^[13, 26–29]; (d) Endothelium-dependent contractions induced by acetylcholine in the rat aorta are accompanied by an increase in cytosolic endothelial calcium concentration^[26, 27] and this increment is greater in preparations of spontaneously hypertensive rats (SHR) compared to those of age-matched

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normotensive Wistar-Kyoto rats (WKY), in line with the larger EDCF-mediated responses in the former^[12, 15, 27]. On the other hand, no significant difference in the increase of calcium concentration in the two strains was observed if the aortae were exposed to A23187^[27].

Phospholipase A₂

The increase in endothelial concentration of the activator ion elicited by agonists such as acetylcholine involves two steps, release of calcium from the sarcoplasmic reticulum followed by influx of extracellular calcium. Acetylcholine binds to the G proteins-coupled muscarinic receptors on the endothelial cell membrane and activates phospholipase C. The latter produces inositol triphosphate which in turn causes the release of calcium from intracellular stores. The resulting calcium-depletion process leads to the production of a messenger termed calcium influx factor [CIF; ^[30]] which displaces the inhibitory calmodulin from the calcium-independent phospholipase A₂ [iPLA₂; ^[31-34]]. Activation of iPLA₂ is an initiating event in the generation of EDCF induced by acetylcholine in the rat aorta^[35]. Activated iPLA₂ produces lysophospholipids which facilitate the opening of store-operated calcium channels (SOCs) leading to the influx of extracellular calcium into the endothelial cells^[34, 36]. This large influx of calcium ions then activates the calcium-dependent phospholipase A₂ (cPLA₂) which converts membrane phospholipids to arachidonic acids, the precursor of prostanoids (Figure 1). That the calcium-dependent form of phospholipase A₂ is crucial for the ultimate production of EDCF is demonstrated by the observation that a specific inhibitor of iPLA₂ does not affect A23187-induced endothelium-dependent contractions, while quinacrine, which inhibits both forms of the enzyme, abolishes the response to both acetylcholine and A23187^[12, 35].

Vitamin D and EDCF

High concentrations of vitamin D appear to have an acute protective effect on endothelial cells by reducing the production of EDCF. Indeed, the *in vitro* administration of 1,25-dihydroxyvitamin D₃ [the most active metabolite of vitamin D^[37]] reduces EDCF-mediated responses induced by acetylcholine but not by the calcium ionophore A23187 in aorta of both SHR and WKY, suggesting that vitamin D acutely reduces EDCF production by an action upstream of the increase in calcium concentration and thus interferes with the calcium surging process (Figure 1)^[26].

Cyclooxygenase

The two isoforms of cyclooxygenase (COX), COX-1 and COX-2, have a comparable ability to catalyze the transformation of arachidonic acid into prostaglandins (Figure 2)^[38]. Both isoforms can play a key role in the generation of EDCF depending on the species, the blood vessel studied and the health conditions of the donor^[2, 3, 19, 28, 39-42]. COX-1 is constitutively expressed in most tissues while COX-2 is inducible^[43, 44]. Early studies demonstrated that non-selective COX inhibitors abolish endothelium-dependent contractions^[13, 17],

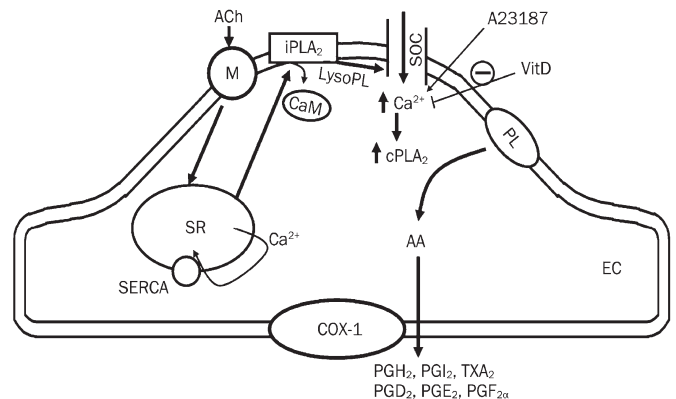


Figure 1. Acetylcholine (ACh) activates muscarinic receptors (M) on the endothelial cell membrane and triggers the release of calcium from intracellular stores. The resulting calcium-depletion process displaces the inhibitory calmodulin (CaM) from iPLA₂. Activated iPLA₂ produces lysophospholipids (LysoPL) which in turn open store-operated calcium channels (SOCs) leading to the influx of extracellular calcium into the endothelial cells. This large influx of calcium ions then activates cPLA₂ which catalyze the production of arachidonic acids (AA). The later is then metabolized by cyclooxygenase-1 (COX-1) to prostanoids. 1,25-Dihydroxyvitamin D₃ (Vit D) acutely reduces endothelium-dependent contraction by inhibiting the calcium surge. cPLA₂=calcium dependent phospholipase A₂; EC=endothelial cells; iPLA₂=calcium independent phospholipase A₂; PGD₂=prostaglandin D₂; PGE₂=prostaglandin E₂; PGF_{2α}=prostaglandin F_{2α}; PGH₂=endoperoxides; PGI₂=prostacyclin; PL=phospholipids; SERCA=sarco/endoplasmic reticulum Ca²⁺-ATPase; SR=sarcoplasmic reticulum; TXA₂=thromboxane A₂.

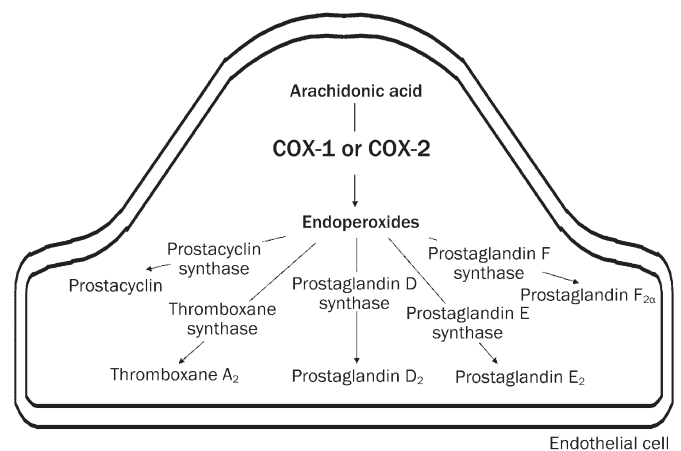


Figure 2. Metabolism of arachidonic acid into specific prostanoids. Arachidonic acid is converted to endoperoxides by the activity of cyclooxygenase (COX). Endoperoxides are then converted to various prostaglandins by their respective synthase.

an observation that has been repeated over the years. Selective inhibitors of COX-1, but not those of COX-2, abrogate endothelium-dependent contractions in the rat aorta^[15, 45-47]. In that preparation, COX-1 is expressed in both endothelial and vascular smooth muscle cells, but the over-expression of this

isoform seen in the SHR aorta is confined to the endothelial cells^[48]. Likewise, bioassay studies demonstrate that only the activation of endothelial COX contributes to the generation of diffusible EDCF in the SHR aorta^[15]. Endothelium-dependent contractions are present in the aorta of COX-2, but not in that of COX-1 knock-out mice^[49]. Taken in conjunction, these findings demonstrated that COX-1 is the preferential constitutive isoform of cyclooxygenase which mediates endothelium-dependent contractions in large arteries of rat and mice. However, with aging or disease, COX-2 can be induced and then contributes to EDCF-mediated responses^[50-53]. By contrast, constitutively expressed COX-2 plays a dominant role in the endothelium-dependent contraction of the hamster aorta irrespective of age^[39].

Prostanoids

Cyclooxygenase converts arachidonic acid into endoperoxides (PGH₂), the intermediate of the prostanoid biosynthesis, which can either act as an EDCF *per se*^[47, 54] or be further transformed into prostacyclin (PGI₂), thromboxane A₂ and various other prostaglandins including prostaglandin D₂ (PGD₂), prostaglandin E₂ (PGE₂) and prostaglandin F_{2α} (PGF_{2α}) by their respective synthases (Figure 2)^[29, 48, 55, 56].

Although PGH₂ has a relatively short half-life and is unstable^[57], it can be a vasoconstrictor EDCF^[29, 47, 48, 55] by activating TP receptors of vascular smooth muscle^[47, 57, 58]. This conclusion is supported by two observations: (a) The aorta of SHR releases more PGH₂ than that of WKY when exposed to acetylcholine^[47]. (b) Similarly to the acetylcholine-induced EDCF-mediated responses, PGH₂-induced contractions in aortae without endothelium are transient and are larger in SHR compared to WKY^[47, 56]. In addition, when tyrosine nitration caused by the local production of peroxynitrite inhibits the activity of prostacyclin synthase^[59], PGH₂ may become even more important in the process.

Prostacyclin is the major cyclooxygenase-derived metabolite of arachidonic acid in endothelial cells^[60]. During endothelium-dependent contractions of rodent aortae in response to acetylcholine, its production is markedly larger than that of other prostaglandins and, together with PGH₂, prostacyclin becomes a major EDCF^[19, 21, 47, 53, 56]. This conclusion is in line with the findings that the gene expression of PGI synthase in the rat aortic endothelial cells is greatly augmented by aging and spontaneous hypertension^[48].

During ADP- and A23187-induced endothelium-dependent contractions, the release of thromboxane A₂ is augmented and an inhibitor of thromboxane A₂ can reduce these contractions, unlike those to acetylcholine^[29, 55, 57]. Therefore, thromboxane A₂ can be regarded as a key EDCF during the EDCF-mediated responses elicited by these agents. Likewise, in certain blood vessels (hamster aorta) or with aging and disease (such as diabetes), an augmented contribution of PGE₂ and PGF_{2α} to EDCF-mediated contractions may become obvious^[39, 61]. This can be explained best by the increased generation of these prostaglandins under conditions of enhanced oxidative stress^[62], in particular as a consequence of the augmented for-

mation of peroxynitrite which inhibits PGI synthase^[59, 63] and diverts arachidonic acid towards PGE₂ and PGF_{2α} synthases^[56]. Obviously, the involvement of individual prostanoids in EDCF-mediated responses varies depending on the species, the blood vessels studied, the endothelium-dependent agonist used, and the age and disease state of the donor.

Reactive oxygen species

Reactive oxygen species (ROS) are generated during a number of normal metabolic activities, but their overproduction leads to oxidative stress which is commonly observed in hypertension, diabetes and atherosclerosis^[20, 64, 65]. During the generation of prostanoids by COX, ROS are formed as by-products. ROS of relevance for endothelium-dependent responses include superoxide anions (O₂⁻), hydroxyl radicals (·OH) and hydrogen peroxide (H₂O₂) (Figure 3). ROS either directly act as EDCF^[66, 67] or indirectly potentiate EDCF-mediated responses by reducing the bioavailability of NO^[68-70] and activating COX in the vascular smooth muscle cells^[16, 18, 71]. This conclusion is based on the following observations: (a) An increased ROS production accompanies acetylcholine- or A23187-induced endothelium-dependent contractions^[27]; (b) Tiron (which scavenges superoxide anions intracellularly) or catalase (which converts hydrogen peroxide to water and oxygen) plus deferoxamine (which prevents the formation of

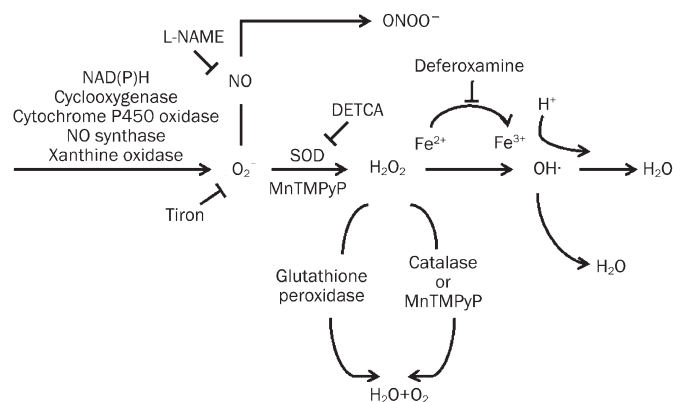


Figure 3. Formation of oxygen-derived free radicals of relevance for endothelium-dependent responses, and pharmacological agents commonly used to determine their importance. Superoxide anions (O₂⁻) can be generated from molecular oxygen by the actions of various enzymes. O₂⁻ can react with NO to form peroxynitrite (ONOO⁻). It can also be converted to hydrogen peroxide (H₂O₂) by superoxide dismutase (SOD). H₂O₂ can be transformed to hydroxyl radicals by ferrous ions or converted to H₂O by catalase and glutathione. Tiron scavenges O₂⁻ inside cells. Deferoxamine is an iron chelator that scavenges hydroxyl radicals. L-NAME inhibits NO synthase. MnTMPyP mimics the combined effect of SOD and catalase. DETCA=diethyldithiocarbamic acid; GSH=glutathione; GSSG=glutathione disulphide; L-NAME=N^ω-nitro-L-arginine methyl ester hydrochloride; MnTMPyP=Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride; NO=nitric oxide; tiron=4,5-dihydroxy-1,3-benzenedisulphonic acid. (Adapted from Shi et al 2007, by permission) Arachidonic acid is converted to endoperoxides by the activity of cyclooxygenase (COX). Endoperoxides are then converted to various prostaglandins by their respective synthase.

hydroxyl radicals) reduce endothelium-dependent contractions in the SHR aorta^[72] and the femoral artery of diabetic rats^[64], suggesting that superoxide anions and hydrogen peroxide augment or even mediate part of the response; (c) ROS formed by the xanthine plus xanthine oxidase reaction elicit contractions of SHR aortae without endothelium which are prevented by both COX inhibitors and TP receptor antagonists, suggesting that the oxygen-derived free radicals stimulate COX in the vascular smooth muscle to produce prostanoids which in turn activate their TP receptors^[18, 72]. (d) ROS increase the degradation of nitric oxide^[68, 69]; (d) Peroxynitrite, a strong cytosolic oxidant generated by the reaction of the superoxide anions and nitric oxide, inactivate PGI synthase^[59, 63] and shifts the production of prostacyclin to that of other vasoconstrictor prostanoids^[56, 73]. (e) In canine basilar arteries, superoxide dismutase (SOD) plus catalase abolish the A23187 induced endothelium-dependent contractions but not the production of prostaglandins and thromboxane A₂ indicating that ROS rather than COX-derived prostanoids are the EDCF in this particular artery^[66]; (f) In the rat pulmonary artery, ROS induce contraction involving the activity of protein kinase C in the vascular smooth muscle^[74]; (g) In vascular smooth muscle of the rat aorta, the ROS-induced calcium sensitization is mediated through the activation of Rho and a subsequent increase in Rho kinase activity^[75], and the latter is crucial in the response to EDCF^[76]; and (h) ROS directly depolarize vascular smooth muscle by inhibiting ATP-sensitive potassium channel (K_{ATP}), voltage-activated potassium channel (K_v) and large conductance calcium-activated potassium channel (BK_{Ca})^[77-79].

Gap junctions

The contact between endothelial and vascular smooth muscle cells is important in the genesis of endothelium-dependent contractions. This conclusion is supported by the observation that the endothelium-dependent contractile response to acetylcholine of layered bioassay ("sandwich") preparations of SHR aortae is much smaller than that of intact aortic rings^[72]. The contraction in the "sandwich preparation" is caused by prostanoids which diffuse across the intracellular gap between the donor (containing endothelial cells) and the recipient strip (without endothelium, responsible for the contraction). Under bioassay conditions, superoxide dismutase plus catalase (both compounds with poor cell permeability) can reduce the acetylcholine-induced endothelium-dependent contractions while they have no effect in intact rings in which tiron inhibits EDCF-mediated responses^[15, 18]. These observations imply that in intact rings, ROS exert their facilitatory effect by either acting in the endothelial cells or being transported from the latter to the vascular smooth muscle cells via preferential channels not accessible to superoxide dismutase. One possible route would be the myoendothelial gap junctions (Figure 4), since the gap junction inhibitor carbenoxolone reduces endothelium-dependent contractions to acetylcholine and the calcium ionophore A23187^[80].

Prostanoid receptors and Rho kinase

Thromboxane-prostanoid receptors (TP-receptors) are the most important prostanoid receptor subtype involved in endothelium-dependent contractions since TP receptor antagonists abolish these responses^[15, 57, 81]. All prostanoids are able to bind with TP receptors, albeit with different affinities^[82]. Thromboxane A₂ is the most potent agonist at TP receptors. Endoperoxides and prostacyclin also activate TP receptors and both of them evoke transient contractions (probably due to their short half-life) which mimic acetylcholine-induced endothelium-dependent contractions^[56]. Binding of EDCF to the TP receptors in turn activates the downstream Rho kinase pathway leading to the increased contractile activity of the vascular smooth muscle^[76].

In the SHR aorta, the gene expression levels and protein presence of TP receptors are not altered, but the responsiveness to endoperoxides is augmented compared to WKY preparations^[47, 48]. This hyperresponsiveness contributes to the prominence of EDCF-mediated responses in the aorta of the SHR. Another crucial aspect in this prominence is that the vascular smooth muscle of aging WKY and of the SHR have lost the ability to respond with relaxation to prostacyclin, despite an unchanged expression of IP receptors and the large production of prostacyclin by endothelial cells exposed to acetylcholine or A23187^[48, 56, 83, 84].

Interactions between NO, EDHF, and EDCF

In the SHR aorta, the concomitant release of NO inhibits endothelium-dependent contractions to acetylcholine^[85, 86], an observation that has led to the systematic use of inhibitors of NO synthases when studying EDCF-mediated responses. In addition, previous exposure to endothelium-derived NO or exogenous NO-donors causes a long-term inhibition of EDCF-mediated responses^[87, 88]. Likewise, in the renal artery of the rat, the absence of EDHF favours the occurrence of endothelium-dependent contractions^[9].

Alternatively, EDCF may also counteract the action of endothelium-derived relaxing factors. Thus, in WKY mesenteric artery, EDHF-mediated relaxations are attenuated by the release of EDCF^[89]. This attenuation is explained best by the EDCF-induced activation of TP-receptors which depolarizes the vascular smooth muscle cells by inhibiting K_v and BK_{Ca}^[90, 91].

Physiological importance

In the early nineteenth century, Bayliss showed that an increase in the internal pressure in the carotid artery of the dog caused its constriction, a seminal observation leading to the concept of autoregulation^[92]. In isolated basilar arteries of the same species, stretch induces a contraction which disappears after the removal of the endothelium, demonstrating an endothelium-dependent process^[14]. This contraction is sensitive to both the COX inhibitor indomethacin and the calcium-influx blocker diltiazem, suggesting that the activity of COX (presumably in the endothelial cells) and the influx of extracel-

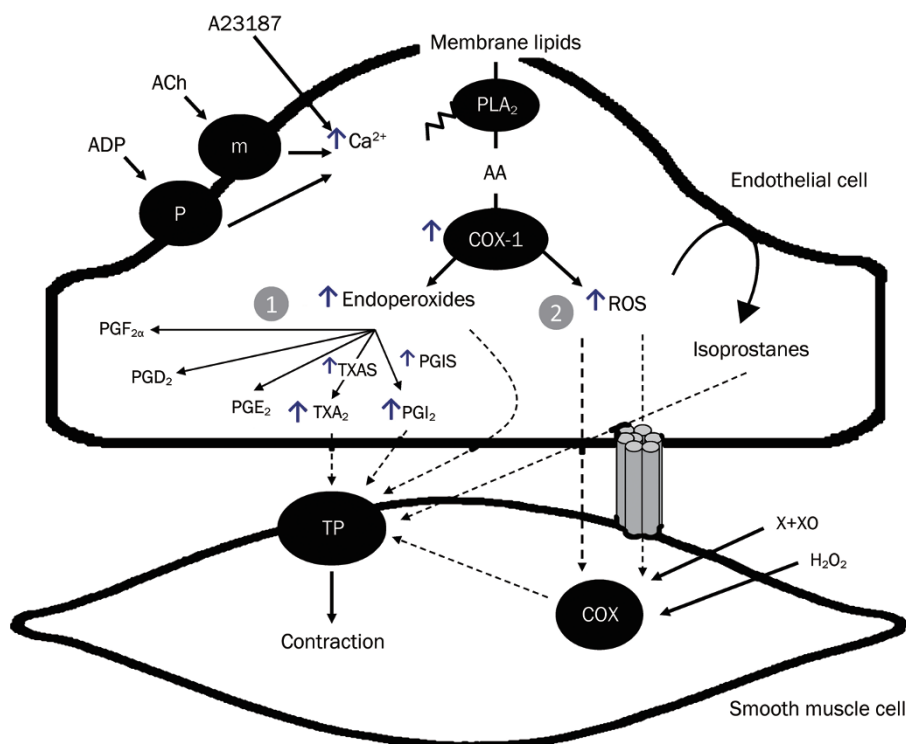


Figure 4. Endothelium-dependent contraction is likely to be comprised of two components: generation of prostanoids and ROS. Each component depends on the activity of endothelial COX-1 and the stimulation of the TP receptors located on the smooth muscle to evoke contraction. In the SHR aorta, there is an increased expression of COX-1 and EP3 receptors, increased release of calcium, ROS, endoperoxides and other prostanoids, which facilitates the greater occurrence of endothelium-dependent contraction in the hypertensive rat. The necessary increase in intracellular calcium can be triggered by receptor-dependent agonists, such as acetylcholine or ADP, or mimicked with calcium increasing agents, such as the calcium ionophore A23187. The abnormal increase in intracellular ROS can be mimicked by the exogenous addition of H_2O_2 or the generation of extracellular ROS by incubation of xanthine with xanthine oxidase. AA=arachidonic acid; ACh=acetylcholine; ADP=adenosine diphosphate; H_2O_2 =hydrogen peroxide; m=muscarinic receptors; P=purinergic receptors; PGD_2 =prostaglandin D₂; PGE_2 =prostaglandin E₂; $PGF_{2\alpha}$ =prostaglandin F_{2 α} ; PGI_2 =prostacyclin; PLA_2 =phospholipase A₂; ROS=reactive oxygen species; TXA_2 = thromboxane A₂; X+XO=xanthine plus xanthine oxidase. (Adapted from Tang and Vanhoutte, 2009, by permission).

lular calcium (presumably in the vascular smooth muscle cells) are required for the active response to stretch^[14]. Likewise, in bovine coronary arteries, stretch elicits an endothelium-independent contraction which requires the activation of NAD(P)H oxidase^[93]. Stretch also directly activates various cation channels on the smooth muscle cells of small arteries facilitating their contraction^[94-96]. Oxygen-derived free radicals play a key role in endothelium-dependent contractions of the canine basilar artery^[66]. Thus, it is tempting to speculate that the endothelium-dependent contraction evoked by stretch (resulting from activation of endothelial COX, the production of ROS and the hypersensitivity of the vascular smooth muscle) may initiate the autoregulatory response, at least in cerebral arteries.

Pathophysiological relevance

As mentioned already, endothelium-dependent contractions are exacerbated by aging, diabetes, hypertension and atherosclerosis^[41, 97, 98]. For example, the blunted endothelium-dependent relaxations in response to acetylcholine in diabetic animals is partly due to the augmented production of EDCF,

resulting from the over-expression and activation of COX and increased ROS production after the chronic exposure of the endothelial cells to high glucose levels^[99]. In essential hypertensive patients, the blunted vasodilatation induced by acetylcholine can almost be normalized by the COX inhibitor indomethacin indicating that COX-derived vasoconstrictors are key players responsible for the abnormal endothelial response^[100]. This indomethacin-sensitive impairment of the response to acetylcholine is accentuated by aging^[98]. However, in secondary hypertension, inhibition of COX does not restore the acetylcholine-induced vasodilatation suggesting that EDCFs are not equally important in all cases of hypertension. It is likely that the prominence of endothelium-dependent contractions observed in arteries of aging and diseased (essential hypertension, diabetes) animals and human reflects the progressive inability of the endothelial cells to generate enough NO to curtail the production of EDCF^[40, 87, 88]. Shifting from the normal release of NO (and EDHF) to that of EDCF likely plays an important role in the development of vascular disease^[40, 101].

Conclusion

Endothelial cells release COX-derived vasoconstrictor prostanoids and reactive oxygen species, which have been termed EDCF. In the SHR, prostacyclin becomes a prominent EDCF acting on TP-receptors, even more so that IP receptor signaling is impaired^[21, 56, 83]. EDCF-mediated responses are amplified in aging normotensive animals (Koga *et al*, 1989; Wong *et al*, 2009), hypertensive^[12] and diabetic^[20, 50, 64, 102] animals. In humans, EDCF plays a role in the endothelial dysfunction that accompanies aging, atherosclerosis, myocardial infarction and essential hypertension^[98, 103, 104].

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