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INVITED REVIEW

Endothelial dysfunction and vascular disease – a 30th anniversary update

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Abstract

The endothelium can evoke relaxations of the underlying vascular smooth muscle, by releasing vasodilator substances. The best-characterized endothelium-derived relaxing factor (EDRF) is nitric oxide (NO) which activates soluble guanylyl cyclase in the vascular smooth muscle cells, with the production of cyclic guanosine monophosphate (cGMP) initiating relaxation. The endothelial cells also evoke hyperpolarization of the cell membrane of vascular smooth muscle (endothelium-dependent hyperpolarizations, EDH-mediated responses). As regards the latter, hydrogen peroxide (H_2O_2) now appears to play a dominant role. Endothelium-dependent relaxations involve both pertussis toxin-sensitive G_i (e.g. responses to α_2-adrenergic agonists, serotonin, and thrombin) and pertussis toxin-insensitive G_q (e.g. adenosine diphosphate and bradykinin) coupling proteins. New stimulators (e.g. insulin, adiponectin) of the release of EDRFs have emerged. In recent years, evidence has also accumulated, confirming that the release of NO by the endothelial cell can chronically be upregulated (e.g. by oestrogens, exercise and dietary factors) and downregulated (e.g. oxidative stress, smoking, pollution and oxidized low-density lipoproteins) and that it is reduced with ageing and in the course of vascular disease (e.g. diabetes and hypertension). Arteries covered with regenerated endothelium (e.g. following angioplasty) selectively lose the pertussis toxin-sensitive pathway for NO release which favours vasospasm, thrombosis, penetration of macrophages, cellular growth and the inflammatory reaction leading to atherosclerosis. In addition to the release of NO (and EDH, in particular those due to H_2O_2), endothelial cells also can evoke contraction of the underlying vascular smooth muscle cells by releasing endothelium-derived contracting factors. Recent evidence confirms that most endothelium-dependent acute increases in contractile force are due to the formation of vasoconstrictor prostanoids (endoperoxides and prostacyclin) which activate TP receptors of the vascular smooth muscle cells and that prostacyclin plays a key role in such responses. Endothelium-dependent contractions are exacerbated when the production of nitric oxide is impaired (e.g. by oxidative stress, ageing, spontaneous hypertension and diabetes). They contribute to the blunting of endothelium-dependent

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vasodilatations in aged subjects and essential hypertensive and diabetic patients. In addition, recent data confirm that the release of endothelin-1 can contribute to endothelial dysfunction and that the peptide appears to be an important contributor to vascular dysfunction. Finally, it has become clear that nitric oxide itself, under certain conditions (e.g. hypoxia), can cause biased activation of soluble guanylyl cyclase leading to the production of cyclic inosine monophosphate (cIMP) rather than cGMP and hence causes contraction rather than relaxation of the underlying vascular smooth muscle.

Keywords cyclic guanosine monophosphate, cyclic inosine monophosphate, endothelin-1, hydrogen peroxide, nitric oxide, prostanoids.

The seminal observation of Robert Furchgott demonstrated that the removal of the endothelial layer from isolated arteries prevents the *in vitro* relaxing response to acetylcholine (Furchgott & Zawadzki 1980). This historical experiment has profoundly modified our thinking about the local control of vascular tone and has been reproduced in different arteries of different species and extended to neurohumoral mediators other than acetylcholine (e.g. De Mey & Vanhoutte 1982, De Mey *et al.* 1982). Bioassay studies convincingly demonstrated that the endothelial cells cause arterial relaxation by releasing a powerful vasoactive substance(s), termed endothelium-derived relaxing factor (EDRF) (Fig. 1). Robert Furchgott's EDRF, because it stimulates soluble guanylyl cyclase in the vascular smooth muscle cells increasing the production of cyclic guanosine monophosphate (cGMP) and is destroyed by superoxide anions, has been identified 30 years ago as nitric oxide (NO) (Furchgott 1988, Ignarro *et al.* 1988, Vanhoutte 2009a, Michel & Vanhoutte 2010, Félétou *et al.* 2012, Toda *et al.* 2012) (Fig. 2). However, the release of NO is not the only way to evoke endothelium-dependent vasomotor changes. Thus, besides NO and, first but not least, prostacyclin (Moncada & Vane 1978), a number of other endothelial mediators and signals can cause endothelium-dependent, NO-independent hyperpolarizations (EDH; Félétou & Vanhoutte 2013) and thus relaxation of the underlying vascular smooth muscle (Fig. 3) (Félétou & Vanhoutte 2009, Shimokawa 2014). Such NO-independent, EDH-mediated responses are prominent in most, but not all smaller arteries. In addition, endothelial cells can release endothelium-derived contracting factors (EDCF), including vasoconstrictor prostanoids (Vanhoutte & Tang 2008, Félétou *et al.* 2010, 2011, Vanhoutte 2011) (Fig. 4), endothelin-1 (De Mey & Vanhoutte 2014), uridine adenosine tetraphosphate (Jankowski *et al.* 2005) and NO itself (Gao & Vanhoutte 2014, Gao *et al.* 2014). When the ability of the endothelial cells to release NO (and to induce EDH) is reduced, and in particular if the propensity to produce EDCF is enhanced, endothelial dysfunction ensues, which appears to be the first step in the chain of events that leads to atherosclerosis and coronary disease. Thus, endothelial dysfunction is the hallmark, and indeed a predictor of cardiovascular disease. This article, at the invitation of the Editor-in-Chief of *Acta Physiologica*, revisits and updates a previous review (Vanhoutte *et al.* 2009) focusing on the role in the genesis of vascular disease of changes in vascular responsiveness due to the imbalance between opposing endothelium-derived mediators, particularly in large arteries. It summarizes the major advances made in the last 7 years as regards the molecular events leading to acute and chronic changes in NO production favouring endothelial dysfunction and alerts the reader to the possibility that NO itself can induce vasoconstriction. This update also emphasizes the further substantiated role of vasoconstrictor endothelium-derived prostanoids, in particular prostacyclin, in such dysfunctions. It highlights the long ignored role of hydrogen peroxide (H_2O_2) as a potent endothelium-derived hyperpolarizing factor. Finally, the review also makes endothelin-1 a potentially important player in the events leading to vascular dysfunction. The authors are aware that whereas the evidence demonstrating beyond doubt the endothelium-dependency of responses discussed below is overwhelming in isolated blood vessels, it is rather scarce *in vivo* (Holtz *et al.* 1984a,b) as the removal of the endothelium in the intact organism is usually incompatible with proper organ function. Hence, when referring to 'endothelium-dependency' *in vivo*, for the sake of clarity, it is implied that responses are meant to stimuli (e.g. acetylcholine, bradykinin, shear stress) which are

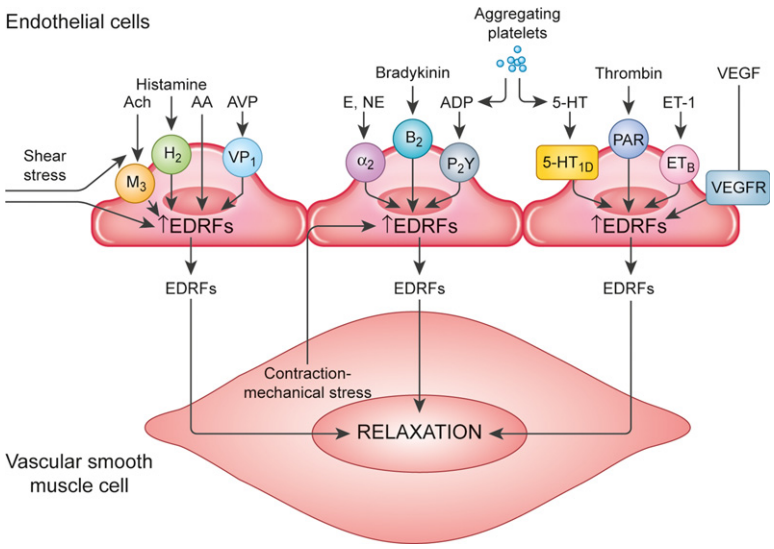

Figure 1 Some of the neurohumoral mediators that cause the release of endothelium-derived relaxing factors (EDRF) through the activation of specific endothelial receptors (circles). A, adrenaline (epinephrine); AA, arachidonic acid; ACh, acetylcholine; ADP, adenosine diphosphate; α, alpha adrenergic receptor; AVP, arginine vasopressin; B, kinin receptor; ET, endothelin, endothelin-receptor; H, histaminergic receptor; 5-HT, serotonin (5-hydroxytryptamine), serotoninergic receptor; M, muscarinic receptor; NA, noradrenaline (norepinephrine); P, purinergic receptor; T, thrombin receptor; VEGF, vascular endothelial growth factor; VP, vasopressin receptor. Several receptors (EP4, IP, Mas, MC1, SIP1, TRPV4 and VDR) are not shown but discussed in the text.

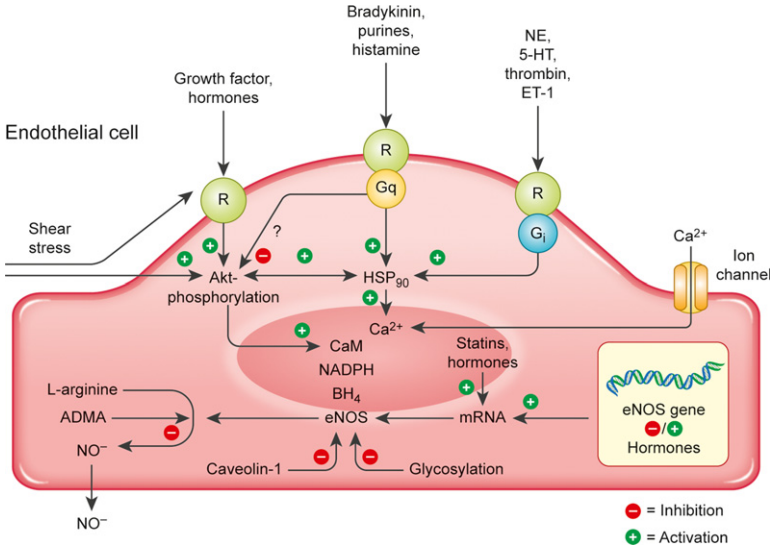

Figure 2 Possible mechanisms by which production of nitric oxide is regulated in endothelial cells. Nitric oxide is produced through enzymatic conversion of L-arginine by nitric oxide synthase (endothelial or type III, eNOS). The transcription of this enzyme is regulated genomically by hormones and growth factors. Stability of eNOS mRNA is modulated by statins and hormones. The enzyme activity of eNOS requires calcium, calmodulin, nicotinamide adenine dinucleotide phosphate and 5, 6, 7, 8-tetrahydrobiopterin (BH_4). Enzyme activity is regulated by complexing of these proteins in microdomains of the endothelial cell. Association with this complex of heat-shock protein 90 (HSP 90) increases enzyme activity. Stimulation of specific receptors on the endothelial surface (R) complexed with guanine nucleotide regulatory proteins [which are sensitive (G_i) or insensitive (G_q) to pertussis toxin] activates intracellular pathways that modulate eNOS activity post-translationally through heat-shock protein 90 or Akt-mediated phosphorylation at Ser1177. Association of eNOS with caveolin-1, phosphorylation at Thr495 or glycosylation of the enzyme reduces activity. A metabolite of L-arginine, asymmetric dimethyl arginine (ADMA) decreases NO production through competitive binding to eNOS; +, indicates stimulation; −, indicates inhibition; ?, indicates those pathways in which the regulation is unknown.

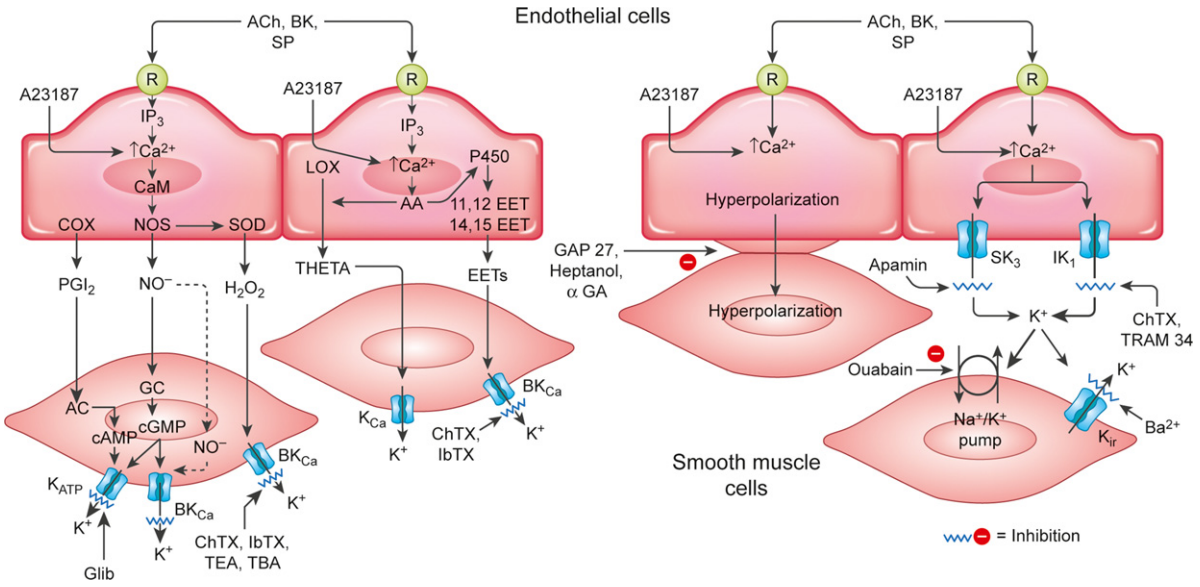

Figure 3 Multiplicity of mechanisms leading to endothelium-dependent hyperpolarization. Substances such as acetylcholine (Ach), bradykinin (BK) and substance P (SP), through the activation of M_3-muscarinic, B_2-bradykinin and NK_1-neurokinin receptor subtypes, respectively, and agents that increase intracellular calcium, such as the calcium ionophore A23187, release endothelium-derived hyperpolarizing factors. CaM, calmodulin; COX, cyclooxygenase; EET, epoxyeicosatrienoic acid; IP3, inositol trisphosphate; GC, guanylate cyclase; NAPE, N-acylphosphatidylethanolamine; NOS, NO synthase; O_2^-, superoxide anions; PGI_2, prostacyclin; P450, cytochrome P450 monooxygenase; R, receptor; X, putative EDHF synthase. SR141716 is an antagonist of the cannabinoid CB, receptor subtype (CB1). Glibenclamide (Glib) is a selective inhibitor of ATP-sensitive potassium-channels (K^+ ATP). Tetraethylammonium (TEA) and tetrabutylammonium (TBA) are non-specific inhibitors of potassium-channels when used at high concentrations (>5 mm), while at lower concentrations (1–3 mm) these drugs are selective for calcium-activated potassium- channels (K^+Ca^{2+}). Iberiotoxin (IBX) is a specific inhibitor of large conductance K^+Ca^{2+}. Charybdotoxin (CTX) is an inhibitor of large conductance K^+Ca^{2+} as well as of intermediate conductance K^+Ca^{2+}(IK^+Ca^{2+}) and voltage-dependent potassium channels. Apamin is a specific inhibitor of small conductance K^+Ca^{2+} (SK^+Ca^{2+}). Barium (Ba^{2+}), in the micromolar range, is a specific inhibitor of the inward rectifier potassium channel (Kir). GAP 27 (an 11-amino acid peptide possessing conserved sequence homology to a portion of the second extracellular loop of connexins), 18a-glycyrrhetinic acid (aGA) and heptanol are gap junction uncouplers.

demonstrated unequivocally to evoke endothelium-dependent relaxations or contractions in isolated blood vessels.

Nitric oxide

Protector of the vascular wall

As such, the endothelium-dependent relaxation to acetylcholine, mediated by activation of M3-muscarinic receptors (Furchgott & Zawadzki 1980, Boulanger *et al.* 1994), is more of pharmacological than of physiological interest. Indeed, few peripheral blood vessels are innervated by cholinergic nerves, the most likely source of acetylcholine. When present, the cholinergic neurones are located in the adventitia, making the access to the endothelial cells rather unlikely. However, leucocytes may provide a physiological source of acetylcholine. In addition, not all isolated blood vessels exhibit endothelium-dependent relaxations in response to acetylcholine, possibly because of the absence of NO synthase traffic inducer (NOSTRIN), which modulates trafficking of M3-receptors and their colocalization with eNOS in endothelial cells (Kovacevic *et al.* 2015); this is the case, for example, in porcine and human coronary arteries in which other stimuli must be employed to evoke such responses (Shimokawa *et al.* 1987, 1991).

Indeed, a number of more physiological stimuli [physical forces, circulating hormones (catecholamines, melanocortin, vasopressin), platelet products (serotonin, adenosine diphosphate), autacoids (histamine, bradykinin, prostacyclin, prostaglandin E_4) and thrombin] share with acetylcholine the ability to elicit endothelium-dependent changes in the tone of the underlying smooth muscle (Fig. 1) (Vanhoutte *et al.* 1986, Shimokawa *et al.* 1988a,b,c, Lüscher & Vanhoutte 1990, Pearson & Vanhoutte 1993, Tang *et al.* 2005b, Ray and Marshall 2006, Stähli *et al.* 2006, Hristovska *et al.* 2007, Levine *et al.* 2007,

Touyz 2007, Rinne *et al.* 2013, Van Langen *et al.* 2013). NO plays a key role in the protection exerted by the endothelium against abnormal constrictions and atherosclerosis of large coronary arteries. Although NO can originate from other sources (Zhao *et al.* 2013), it is produced mainly by the constitutive isoform of NO synthase (eNOS, NOS III), which can be activated (phosphorylated) in both Ca^{2+}-dependent and Ca^{2+}-independent ways (Fig. 2) (Marletta 1989, Schini-Kerth & Vanhoutte 1995, Moncada 1997, Li *et al.* 2002a, Dudzinski *et al.* 2006, Feron & Balligand 2006, O'Rourke *et al.* 2006, Bauer and Sotníková 2010, Michel & Vanhoutte 2010, Maron & Michel 2012, Toda *et al.* 2012). NO not only prevents abnormal constriction (vasospasm) of the coronary arteries, which favours intraluminal clot formation, but also inhibits the aggregation of platelets, the expression of adhesion molecules at the surface of the endothelial cells, and hence the adhesion and penetration of white blood cells (macrophages), and the release and action of the vasoconstrictor and mitogenic peptide endothelin-1 (ET-1) (Fig. 5). The protective release of NO is triggered by the local presence of thrombin and substances released by aggregating platelets. When this protective role of NO is curtailed, the inflammatory response (Ross 1999) that leads to atherosclerosis is initiated (Vanhoutte 1988, 1996, 1997, 2000, 2002, 2009b, Lüescher *et al.* 1993, Li *et al.* 2002b, Vallance 2003, Cooke 2004, Voetsch *et al.* 2004, Félétou & Vanhoutte 2006b, Vanhoutte *et al.* 2009).

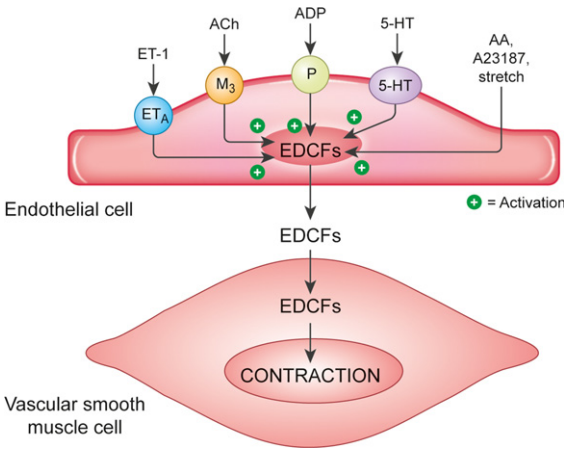

Figure 4 Under certain conditions, the endothelial cells, when activated by neurohumoral mediators, subjected to sudden stretch or exposed to the Ca^{2+} ionophore A23187, release vasoconstrictor substances, termed endothelium-derived contracting factor(s) (EDCFs), which diffuse to the underlying vascular smooth muscle and initiates its contraction. AA, arachidonic acid; ACh, acetylcholine; ADP, adenosine diphosphate; ET, endothelin; 5-HT, 5-hydroxytryptamine; M, muscarinic receptor; P, purinoceptor; O, membrane receptors.

The role played by the endothelial cells to protect against thrombin and platelet products by increasing the activity of eNOS has been demonstrated both *in vitro* (De Mey *et al.* 1982, Cohen *et al.* 1983, 1984, Houston *et al.* 1985, 1986, Shimokawa *et al.* 1988a, Derkach *et al.* 2000, Motley *et al.* 2007, Touyz 2007) and *in vivo* (Shimokawa & Vanhoutte 1991). Serotonin (5-hydroxytryptamine, 5HT) and adenosine diphosphate (ADP) are the two mediators released by aggregating platelets that can activate eNOS and thus augment the production of NO. Serotonin is the most important and stimulates 5-HT_{1D} serotonergic receptors of the endothelial cell membrane. ADP is a relatively minor contributor that acts on P_{2y} purinoceptors (Fig. 5). The serotonergic receptors and those for thrombin are coupled to the activation of eNOS through pertussis toxin-sensitive G_i proteins, while the P_{2y} purinoceptors are linked to the enzyme

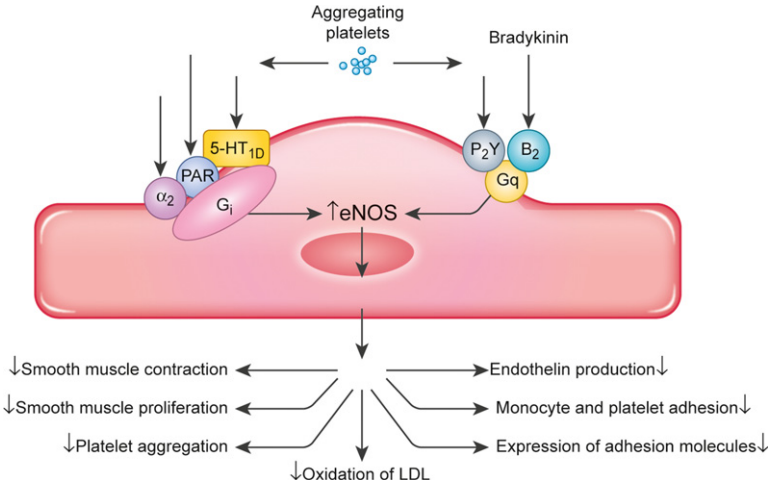

Figure 5 G-protein-mediated signal transduction processes in a normal, native endothelial cell. Activation of the cell causes the release of nitric oxide (NO), which has important protective effects in the vascular wall. 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G, coupling proteins.

by G_q proteins (Flavahan *et al.* 1989, Shimokawa *et al.* 1991, Flavahan & Vanhoutte 1995). If the endothelium is absent or dysfunctional, such relaxations are no longer observed, and aggregating platelets induce constrictions (vasospasm), because they release the powerful vasoconstrictors thromboxane A_2 and serotonin.

The physiological importance of the endothelium-dependent relaxations to platelet products is obvious (Vanhoutte 1988, 1996, 1997, 2002, 2009b, Félétou & Vanhoutte 2006b, Vanhoutte *et al.* 2009). Thus, if platelet aggregation occurs in a coronary artery with a healthy endothelium, the release of serotonin (and ADP) by the platelets and the local production of thrombin will stimulate the endothelial cells to release NO. The endothelial mediator will cause the underlying smooth muscle to relax, thus increasing blood flow and mechanically impeding the progression of the coagulation process. NO also exerts in synergy with prostacyclin an immediate feedback inhibition on the platelets (Radomski *et al.* 1987). When the endothelial barrier is damaged by injury, the aggregating platelets can reach the vascular smooth muscle cells, and cause their contraction by releasing thromboxane A_2 and serotonin, initiating the vascular phase of hemostasis. The endothelium-dependent response to aggregating platelets is not present to the same extent in all arteries, but is most prominent in the coronary and cerebral circulations.

NO, the gate keeper. Besides its direct role as a vasodilator, NO also modulates the release of other endothelium-derived mediators. Thus, in a number of larger arteries, EDH-mediated relaxations/dilatations become prominent only when the synthesis of NO is inhibited (Olmos *et al.* 1995), illustrating the gate-keeping role of the latter (Félétou *et al.* 2011). Hence, EDH is able to take over, at least temporarily, in the case of 'classical' endothelial dysfunction associated with a loss of NO synthesis [e.g. in arteries with regenerated endothelium (Thollon *et al.* 2002) or in eNOS-deficient mice (Brandes *et al.* 2000)], demonstrating strong compensatory efficiency of EDH-mediated responses. Intriguingly, exogenous NO attenuates EDH-mediated responses in porcine coronary arteries *in vitro* (Bauersachs *et al.* 1996) and in the canine coronary circulation *in vivo* (Nishikawa *et al.* 2000) and NO has a negative feedback effect on endothelium-dependent relaxation through cGMP-mediated desensitization in isolated canine coronary arteries (Olmos *et al.* 1995). Indeed, clinical studies show that chronic therapy with nitrate, used as a NO donor, in patients with ischaemic heart disease does not yield a benefit on mortality (Kojima *et al.* 2007, Ambrosio *et al.* 2010), confirming the importance of the physiological balance between NO and EDH in the coronary circulation. Likewise, the amount of NO formed in the endothelial cells controls the release of vasoconstrictor prostanoids (see section The major villains: endothelium-derived vasoconstrictor prostanoids) and ET-1 (see section Endothelin-1).

Modulation of the protective role of nitric oxide

The ability of the endothelium to release NO can be upregulated or downregulated in the intact organism by a number of chronic factors.

Upregulation. Shear stress—Both acute and chronic increases in flow, and the resulting increase in shearing force (shear stress) of the blood on the endothelial cells, augment the expression and the activity (in a Ca^{2+}-independent way) of eNOS, and thus the release of EDRF/NO (Fig. 2), although EDH-mediated responses can contribute (Rubanyi *et al.* 1986, Miller & Vanhoutte 1988, Davis *et al.* 2001, Stepp *et al.* 2001, Bellien *et al.* 2006, Yan *et al.* 2007). The immediate effect of an increase in shear stress on the release of NO explains flow-mediated dilatation (FMD), a phenomenon often used to estimate the functional state of the endothelium in humans. However, there are several limitations when equating flow-mediated vasodilatation with the release of NO, particularly in humans. First, special care must be taken to limit variability and insure reproducibility (Charakida *et al.* 2013). Second, in patients, products of cyclooxygenase (Nohria *et al.* 2014), hydrogen peroxide (H_2O_2; Kang *et al.* 2011b, Freed *et al.* 2014) or other EDH-mediators (Nohria *et al.* 2014) may contribute to the response to increases in shear stress. Third, one should always consider the possibility that reductions in responses to shear stress or endothelium-dependent vasodilator agents can be due to the concomitant release of endothelium-derived vasoconstrictors (see sections The major villains: endothelium-derived vasoconstrictor prostanoids and Endothelin-1), or to a reduced (e.g. Kim *et al.* 1992, Schjerning *et al.* 2013) or abnormal (see section Hypoxia: when NO turns bad) responsiveness of the vascular smooth muscle cells to NO. Thus, when observing changes in flow-mediated vasodilatation, appropriate pharmacological experiments (in particular using inhibitors of cyclooxygenases and NO synthases) must be performed before attributing the observed differences to altered NO bioavailability.

The *acute* effect of shear stress in increasing NO release involves several mechanisms (Davies 1995, Busse & Fleming 2003, Chiu & Chien 2011, Liu *et al.* 2013, Fleming 2015, Sun & Feinberg 2015): (i) In the porcine and human coronary circulations, the local

production of bradykinin that stimulates the release of NO through a G_q-dependent mechanism (Fig. 6) (Flavahan *et al.* 1989, Mombouli & Vanhoutte 1991, 1995, Shimokawa *et al.* 1991, Roves *et al.* 1995). In rat carotid arteries, the flow-mediated increase in local bradykinin production requires first the activation of angiotensin II AT2 receptors (Bergaya *et al.* 2004), possibly stimulating prolylcarboxypeptidase plasma prekallikrein activator (Zhu *et al.* 2012); (ii) lectin–oligosaccharide interactions resulting in sensitization of G protein-coupled receptors of the endothelial cell membrane (Perez-Aguilar *et al.* 2014); (iii) Immediate activation of a K^+ current through KCa channels, inducing an increased NO release (Olesen *et al.* 1988, Ohno *et al.* 1993). This is followed short-term by upregulation of endothelial calcium-activated potassium channels ($KCa_{2.3}$ and $KCa_{3.1}$) via a calcium-/calmodulin-dependent protein kinase (Takai *et al.* 2013); (iv) activation of endothelial transient receptor potential (TRP) receptors leading to increased calcium influx and stimulation of eNOS (as well as initiation of EDH-mediated relaxation) (Olesen *et al.* 1988, Mendoza *et al.* 2010, Bubolz *et al.* 2012); and (v) caveolae-dependent modulation of endothelial signal transduction from shear stress to NO production and release (Chai *et al.* 2013). However, the shear stress-induced increase in NO release can be blunted by the simultaneous release of hydrogen sulphide (H_2S) (Chai *et al.* 2015).

The *chronic* effect of shear stress is due to the upregulation of eNOS, whereby tyrosine kinase c-Src accelerates both the transcription and activation (phosphorylation) of the enzyme, leading to a larger release of NO for each given stimulation, explaining the beneficial effects of regular exercise on endothelial function (Miller & Vanhoutte 1988, Mombouli *et al.* 1996, Davis *et al.* 2001, Hambrecht *et al.* 2003, Suvorava *et al.* 2004, Watts *et al.* 2004, Lauer *et al.* 2005, Gertz *et al.* 2006, Rakobowchuk *et al.* 2008, Tarhouni *et al.* 2013, 2014, Bender & Laughlin 2015). The chronic impact of shear stress on eNOS involves transforming growth factor-β (TGF-β) and the subsequent activation of Krüppel-like factor 2 (KLF2) (Davies *et al.* 2013, Walshe *et al.* 2013, Doddaballapur *et al.* 2015). It is modulated by the endothelial level of bactericidal permeability increasing fold containing family B member 4 (BPIFB4) (Villa *et al.* 2015).

Temperature—Moderate cooling acutely causes relaxations of isolated arteries (canine coronary, femoral and renal; rat aorta and superior mesenteric) which are endothelium-dependent and involve the activation of eNOS with the subsequent production of NO (Evora *et al.* 2007, Zou *et al.* 2015). Logically, this response is due to stimulation by cold of TRP channels, well known to react to changes in temperature (Venkatachalam & Montell 2007). In arteries of normotensive rats, the TRP channel involved appears to be the transient receptor potential cation channel subfamily A member 1 (TRPA1) subtype; however, in those of spontaneously hypertensive rats (SHR), the TRPA1-mediated response is absent and is compensated by the activation of transient receptor potential

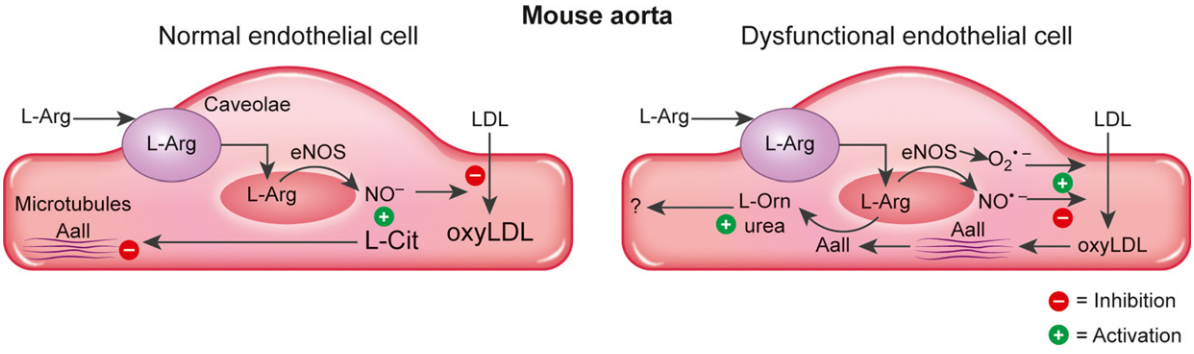

Figure 6 Model of endothelial dysfunction in the hypercholesterolemic mouse. *Left*, In the normal mouse aortic endothelium, L-arginine (L-Arg) is transformed by eNOS to NO, which exerts its well-documented beneficial effects (most are not shown for the sake of clarity), including inhibition of the oxidation of LDLs to OxyLDL. The by-product of the reaction, L-citrulline (L-Cit), inhibits arginase II (AaII), which is constrained to the microtubules (MT). *Right*, in the aortic endothelium of the $ApoE^{-/-}$ and the wild-type hypercholesterolaemic mice, the accumulation of OxyLDL dislocates arginase II from the microtubules and augments its activity. Arginase II competes with endothelial NO synthase for the common substrate L-arginine, leading to uncoupling of NO synthase and the production of superoxide anions (O_2^-), which further enhance the production of OxyLDL. The latter also facilitates dissociation of eNOS from the caveolae and reduces the genomic expression of the enzyme, leading to further reduction in the production of NO. This model does not account for the biological effects, if any, of L-ornithine (L-Om) and urea produced by arginase II. It also does not account for endothelium-derived relaxing signals other than NO, or for the generation of endothelium-derived contracting substances. CM indicates cell membrane; +, facilitation; −, inhibition.

vanilloid type 4 (TRPV4) channels (Zou *et al.* 2015). Activation of the latter channels also contributes to flow-mediated dilatations (Mendoza *et al.* 2010, Bubolz *et al.* 2012). Surprisingly, the cold-induced, endothelium-dependent relaxations of canine and SHR arteries are prevented by the muscarinic antagonist atropine (Evora *et al.* 2007, Zou *et al.* 2015), implying a role for locally produced acetylcholine, the prototypical inducer of endothelium-dependent relaxations mediated by activation of M3-muscarinic receptors (Furchgott & Zawadzki 1980, Boulanger *et al.* 1994, Kovacevic *et al.* 2015). It has long be suspected and even demonstrated that endothelial cells, also of human origin, contain all the ingredients necessary to produce and metabolize acetylcholine (Parnavelas *et al.* 1985, Olesen *et al.* 1988, Milner *et al.* 1989, 1990, Lan *et al.* 1996, Haberberger *et al.* 2002, Kirkpatrick *et al.* 2003, Lips *et al.* 2003, Sandow *et al.* 2012). The activation of an endothelial non-neuronal cholinergic system acting in an autocrine manner to induce endothelium-dependent relaxation as seen with moderate cooling (Evora *et al.* 2007, Zou *et al.* 2015) finally may provide a physiological role for the endothelial muscarinic receptor discovered early by Robert Furchgott, but long thought to be a pharmacological curiosity.

Arginine—In the human, the NO precursor L-arginine (Figs 2 and 6) is a semi-essential amino acid as it can be synthesized *de novo* from L-citrulline. Therefore, although decreased availability of L-arginine and L-citrulline can contribute to NO deficiency (Getz & Reardon 2006, El-Hattab *et al.* 2012) and the *acute* administration of L-arginine can favour endothelium-dependent responses in humans (Bode-Bőger *et al.* 1996, Taddei *et al.* 1997a, Perticone *et al.* 2005), its *chronic* supplementation offers little therapeutic benefit in patients with vascular disease (Wilson *et al.* 2007), reinforcing the early suspicion (Schini & Vanhoutte 1991a) that the semi-essential amino acid is rarely a limiting factor for the endothelial production of NO. Exceptions may be when the endothelial arginases, which compete with eNOS for this substrate, are more active (Fig. 6) (Ming *et al.* 2004, Johnson *et al.* 2005, Brandes 2006, Ryoo *et al.* 2006, 2008, Holowatz & Kenney 2007, Katusic 2007, Santhanam *et al.* 2007, Romero *et al.* 2008, Vanhoutte 2008, Chandra *et al.* 2012, El-Bassossy *et al.* 2012, Yao *et al.* 2013) or when the L-arginine transporter (cationic amino acid transporter 1, CAT-1) is deficient (Martens *et al.* 2014), although the extra- rather than the intracellular concentration of the precursor may be critical for a sufficient supply to eNOS (Shin *et al.* 2011). To judge from cell culture studies, pronounced exposure to arginine may even accelerate endothelial senescence (Scalera *et al.* 2009a).

Tetrahydrobiopterin—The biosynthesis of tetrahydrobiopterin (BH4), an essential cofactor for NO formation by eNOS (Fig. 2), from sepiapterin is catalysed by GTP-cyclohydrolase I (GTPCH I; Kang *et al.* 2011a, Meininger & Wu, 2011, Zhang *et al.* 2011b). *Chronically* low circulating levels of BH4 are accompanied by reduced endothelium-dependent relaxations (Moreau *et al.* 2012, Zhang *et al.* 2012c). Likewise, BH4 deficiency, caused by mutation or deletion of this enzyme, results in reduced NO-mediated and endothelium-dependent relaxations which can be reversed by the administration of sepiapterin (Chuaiphichai *et al.* 2014, d'Uscio *et al.* 2014). The decreased production of NO is compensated in part by the generation of H_2O_2 by the uncoupled eNOS (Chuaiphichai *et al.* 2014; see section Endothelium-derived hydrogen peroxide). Supplementation with BH4 improves endothelial function in hypertensive animals as well as in post-menopausal women (Kang *et al.* 2011a, Moreau *et al.* 2012).

Gender and sex hormones—Sex hormones *chronically* affect the function of endothelial cells. Thus, endothelium-dependent relaxations are more pronounced in arteries from pre-menopausal female than male animals (Kauser & Rubanyi 1995, Kähönen *et al.* 1998, Dantas *et al.* 2004, Levy *et al.* 2009, Zuloaga *et al.* 2014). Likewise, to judge from the reduced flow-mediated vasodilatation, endothelial responsiveness is blunted in post-menopausal women, a condition which is due in part to BH4 deficiency (Moreau *et al.* 2012), and the development of endothelial dysfunction is less prominent in pre-menopausal women than in age-matched men and post-menopausal women, highlighting the protective effect of oestrogens (Taddei *et al.* 1996, Perregaux *et al.* 1999, Harris *et al.* 2012, Moreau *et al.* 2012). In the animal, ovariectomy *per se* does not alter or even increases the mRNA expression and the presence of eNOS (Wassmann *et al.* 2001, Okano *et al.* 2006). The *chronic* reintroduction of physiological levels of *oestrogens*, and the resulting activation of endothelial estrogen receptor α (ERα) in ovariectomized animals augments endothelium-dependent relaxations (Gisclard *et al.* 1988, Wassmann *et al.* 2001, Sakuma *et al.* 2002, Santos *et al.* 2004, Nawate *et al.* 2005, Scott *et al.* 2007, Kang *et al.* 2011b, Chan *et al.* 2012, Tarhouni *et al.* 2013, 2014, Costa *et al.* 2015), favours blood flow-mediated remodelling of resistance arteries (Tarhouni *et al.* 2013) and accelerates endothelial healing after injury (Filipe *et al.* 2008). Although the endothelial effects of oestrogen involves mainly activation of eNOS

(Fig. 2) and a greater production of NO, ERα stimulation also can augment the production of prostacyclin (Jobe *et al.* 2013) and EDH-mediated responses (Liu *et al.* 2001, 2002, Sakuma *et al.* 2002, Nawate *et al.* 2005, Luksha *et al.* 2006, Chan *et al.* 2012, Wong *et al.* 2014, Yap *et al.* 2014, Kong *et al.* 2015). The *acute* and *chronic* potentiating effect of oestrogens on endothelium-dependent relaxations involve both genomic (Fig. 2) and non-genomic effects (Tostes *et al.* 2003, Keung *et al.* 2005, Miller & Duckles 2008). It depends presumably on both a reduction in oxidative stress leading to an increased bioavailability of endothelium-derived NO and an increased responsiveness of the vascular smooth muscle cells to vasodilator stimuli (Wassmann *et al.* 2001, Han *et al.* 2007, Li *et al.* 2007a, Scott *et al.* 2007, Costa *et al.* 2015, Mazzuca *et al.* 2015). In the intact organism, a reduced production of the endogenous inhibitor of eNOS, asymmetric dimethyl arginine (ADMA), can contribute to the improvement to the improvement of endothelial function by estrogens (Monsalve *et al.* 2007). The *chronic* improvement of endothelium-dependent relaxation by oestrogens involves silent information regulation 2 homologue (SIRT1) and AMP-activated protein kinase (AMPK) (Schulz *et al.* 2005, Liao *et al.* 2011, Bendale *et al.* 2013, Yang & Wang 2015). Phytoestrogens and selective estrogen receptor modulators (SERMs) also *acutely* potentiate endothelium-dependent relaxations/vasodilatations (Lee & Man 2003, Sbarouni *et al.* 2003, Wong *et al.* 2006, Chan *et al.* 2007, Leung *et al.* 2007). In coronary arteries, the potentiating effect of *chronic* treatment with oestrogens is observed only with stimuli that activate G_i-coupled receptors on the endothelial cells. Although EDH-mediated dilatations also are more pronounced in females than in males (Liu *et al.* 2001, 2002, Sakuma *et al.* 2002, Nawate *et al.* 2005, Luksha *et al.* 2006, Morton *et al.* 2007, Sun *et al.* 2011, Chan *et al.* 2012, Wong *et al.* 2014, Yap *et al.* 2014), it is likely that the potentiating effect of oestrogens on NO release (presumably resulting from lower oxidative stress) helps to explain why endothelium-dependent relaxations are more pronounced in arteries from female than male animals and thus why women are protected against coronary disease, at least until the age of menopause.

Insulin—Insulin *acutely* facilitates and even causes NO-dependent vasodilatations *in vivo* and *in vitro* (Steinberg *et al.* 1994, Taddei *et al.* 1995b, Lembo *et al.* 1997a, Potenza *et al.* 2006, Subramaniam *et al.* 2009, Genders *et al.* 2011, Nemoto *et al.* 2011, Meijer *et al.* 2013, Jang *et al.* 2013, Osto *et al.* 2015). In isolated blood vessels, it selectively enhances G_i protein-mediated responses (Lembo *et al.* 1997b). Insulin enhances the expression of eNOS in native endothelial cells *in vitro* (Fisslthaler *et al.* 2003) and stimulates the phosphorylation of the enzyme (Jang *et al.* 2013, Tassone *et al.* 2013).

Angiotensin (1–7)—The heptapeptide angiotensin (1–7) is formed from angiotensin II by angiotensin-converting enzyme 2 (ACE2) and activates Mas receptors (Ferreira & Santos 2005, Carey 2013, Raffai *et al.* 2014). In humans, flow-mediated vasodilatation correlates positively with the circulating levels of angiotensin (1–7) (Sullivan *et al.* 2015). In mice, deletion of ACE2 results in blunted flow-mediated dilatations (Patel *et al.* 2012). In isolated arteries, both stimulation of ACE2 (using a small molecule activator of the enzyme *in vitro*) and exogenous angiotensin (1–7) *acutely* potentiate endothelium-dependent relaxations, in particular those evoked by bradykinin [because the heptapeptide inhibits angiotensin-converting enzyme (ACE1) which is the major contributor to the degradation of the kinin] (Tom *et al.* 2001, Raffai *et al.* 2011, 2014, Fraga-Silva *et al.* 2013). However, no potentiation of vasodilator responses to bradykinin is observed in the human forearm (Wilsdorf *et al.* 2001). *Chronic* administration of angiotensin (1–7) restores NO-mediated, endothelium-dependent dilations to acetylcholine in arteries of animals fed on a high-salt diet, an effect attributable to Mas receptor activation and reduced oxidative stress (Durand *et al.* 2010, Raffai *et al.* 2011, Shenoy *et al.* 2014). Likewise, *chronic* administration of a small molecule activator of ACE2 reduces the endothelial dysfunction in hypertensive and diabetic animals (Fraga-Silva *et al.* 2013, Shenoy *et al.* 2013, 2014).

Vascular endothelial growth factor—Vascular endothelial growth factor (VEGF) can stimulate/upregulate eNOS (Fig. 1) and the major *chronic* side effect of its inhibitors is the occurrence of hypertension, suggesting a physiological role for the growth factor in maintaining normal endothelial control of vasomotor tone (Facemire *et al.* 2009, Zhang *et al.* 2011a, Hou *et al.* 2012, Skinner *et al.* 2014). In humans, the effect of VEGF inhibitors is complex as they do not affect flow-mediated dilatations but reduce the response to acetylcholine (Mayer *et al.* 2011, Thijs *et al.* 2013). In pigs and humans, the hypertensive response to VEGF inhibitors may involve increased production of ET-1 (see section Endothelin-1) rather than modulation of NO release (Kappers *et al.* 2012, Lankhorst *et al.* 2014).

Vitamin D—Chronic vitamin D insufficiency is associated with reduced flow-mediated vasodilatation in

humans (Al Mheid *et al.* 2011, Jablonski *et al.* 2011, Sokol *et al.* 2012). Likewise, endothelium-specific deletion of the vitamin D receptor (VDR) blunts endothelium-dependent relaxations in the mouse (Ni *et al.* 2014). Conversely, vitamin D supplementation improves endothelial function in patients with kidney disease (Zoccali *et al.* 2014). The improvement of endothelial function is attributable to increased expression/activity of eNOS and a greater production of NO together with inhibition of the increase in oxidative stress caused by endogenous angiotensin II (Martínez-Miguel *et al.* 2014, Ni *et al.* 2014, Schulz *et al.* 2014). In addition, both *acute* and *chronic* administration of vitamin D reduce prostanoid-mediated, endothelium-dependent contractions *ex vivo* (Wong *et al.* 2008, 2010b; see section The major villains: endothelium-derived vasoconstrictor prostanoids). However, the positive effect of vitamin D on endothelial function can be offset by upregulation of the production or action of ET-1 (Absi & Ward 2013, Martínez-Miguel *et al.* 2014; see section Endothelin-1).

Adiponectin—Most blood vessels are surrounded by a variable amount of peri-vascular adipose tissue (PVAT), originally thought to provide mechanical support for the blood vessel and serve as an energy reserve. PVAT has been routinely removed in traditional studies on isolated blood vessels. However, it can modulate vascular function (Gu & Xu 2013, Brown *et al.* 2014b, Withers *et al.* 2014, Oriowo 2015). Upon stimulation by a variety of agonists or electrical stimulation, PVAT can *acutely* alter the tone of the vascular smooth muscle that it surrounds by releasing adipocyte-derived relaxing factor(s) (ADRF) (Meyer *et al.* 2013, Withers *et al.* 2014, Oriowo 2015). These adipocyte-derived factors, referred to as *adipokines* (modulating proteins acting locally in an autocrine/paracrine fashion or systemically as hormones), are transferable in various arterial preparations and also in veins (Lu *et al.* 2011a, Gollasch 2012). The candidates proposed as ADRF include adiponectin, angiotensin 1–7, H_2S, leptin, methyl palmitate, NO, omentin, prostacyclin and visfatin (Gollasch 2012, Gu & Xu 2013, Oriowo 2015). The exact chemical nature of ADRF varies depending on the vascular bed and animal of interest. Of those, adiponectin has received the most attention (Lynch *et al.* 2013b, Margaritis *et al.* 2013, Weston *et al.* 2013). Peroxidation products formed in the vascular wall upregulate adiponectin gene expression in PVAT via a peroxisome proliferator-activated receptor-γ ($PPAR_\gamma$)-dependent mechanism (Margaritis *et al.* 2013). The release of adiponectin can become disturbed in animals and patients with hypertension, obesity and/or metabolic syndrome (Potenza *et al.* 2006, Gollasch 2012, Gu & Xu 2013, Meyer *et al.* 2013, Oriowo 2015). In addition, adiponectin has long been identified as a *chronic* insulin sensitizer influencing glucose and fat metabolism, but the adipokine also *acutely* exerts direct actions on the blood vessel wall (Hui *et al.* 2012, Xu & Vanhoutte 2012, Meijer *et al.* 2013). It associates with T-cadherin and binds to adiponectin receptors 1 and 2 to moderate endothelial dysfunction (Xu & Vanhoutte 2012, Parker-Buffen *et al.* 2013). The adipokine does so by inhibiting inflammatory kinase Jun NH2-terminal kinase and reducing the production of reactive oxygen species (ROS), promoting the coupling and activity of eNOS, increasing the bioavailability of both BH4 and NO, suppressing endothelial cell activation and apoptosis and promoting endothelial repair (Chen *et al.* 2003, Hattori *et al.* 2003, Tan *et al.* 2004, Cheng *et al.* 2007, Li *et al.* 2007b, Wang & Scherer 2008, Zhu *et al.* 2008, Margaritis *et al.* 2013, Meijer *et al.* 2013, Liu *et al.* 2014c, Zhi *et al.* 2014). Epidemiological studies in different ethnic groups have identified *chronic* adiponectin deficiency (hypo-adiponectinaemia) as an independent risk factor for endothelial dysfunction, hypertension, coronary heart disease, myocardial infarction and other cardiovascular complications (Zhu *et al.* 2008, Azuma *et al.* 2015). Hypo-adiponectinaemia *per se* (independent of diabetes) is associated with impaired NO-mediated, endothelium-dependent vasodilatations (Zhu *et al.* 2008). Conversely, elevations of the circulating levels of adiponectin (hyperadiponectinaemia) by genetic, dietary or pharmacological approaches alleviate various vascular dysfunctions (Zhu *et al.* 2008, Liu *et al.* 2014c).

Other hormones—Erythropoietin (EPO) reduces oxidative stress and facilitates NO production (and also that of H_2O_2; see section Endothelium-derived hydrogen peroxide) and thus prevents endothelial dysfunction resulting from eNOS uncoupling both *in vivo* and *in vitro* (Yada *et al.* 2010, Kuriyama *et al.* 2014, d'Uscio *et al.* 2014). *Glucagon-like peptide-1* (GLP-1) not only enhances the vasodilator response to acetylcholine (Basu *et al.* 2007) but also *acutely* evokes endothelium-dependent hyperpolarizations and relaxations (Osto *et al.* 2015, Salheen *et al.* 2015) and augments microvascular recruitment in a NO-dependent fashion (Dong *et al.* 2013). The peptide improves endothelium-dependent, NO-mediated relaxations (Osto *et al.* 2015) and upregulates the activity and protein expression of eNOS in human endothelial cells (Ding & Zhang 2012). *Chronic* inhibition of the enzyme responsible for its breakdown, dipeptidyl peptidase-4 (DPP4), corrects endothelial dysfunction

(Liu *et al.* 2012b, Matsubara *et al.* 2012, Salheen *et al.* 2015). In cultured human endothelial cells, *melanocortin* (α-melanocyte-stimulating hormone, MSH) activates melanocortin 1 (MC1) receptors leading to the increased expression and phosphorylation of eNOS. In mice, *in vivo* treatment with a stable MSH analog ameliorates the endothelial dysfunction associated with ageing and diet-induced obesity (Rinne *et al.* 2013). In the aorta of recessive yellow mice deficient in MC1 signalling, contractile capacity and NO-dependent relaxations are impaired and arterial stiffness is increased (Rinne *et al.* 2015). In addition, humans with weak MC1 function exhibit reduced flow-mediated dilatations and increased arterial stiffness (Rinne *et al.* 2015). These observations suggest a *chronic* physiological endothelial protective role of the hormone. *Thyroid hormone* upregulates eNOS and augments the endothelial production of NO in the animal (Spooner *et al.* 2004). Hyperthyroidism in the rat is accompanied by augmented relaxations to acetylcholine (Deng *et al.* 2010), but as the contractions to phenylephrine are reduced and relaxations to sodium nitroprusside are potentiated, it is uncertain whether or not this reflects true facilitation of endothelium-dependent relaxations. However, conditional selective overexpression of thyroid hormone receptors ($TR\alpha_1$) in endothelial cells of the mouse activates the eNOS pathway and protects the heart against injury after an ischaemic insult (Suarez *et al.* 2014). In arteries of young, but not aged rodents, *relaxin*, which plays a physiological role mainly during pregnancy, augments NO bioavailability in virtue of its antioxidant properties and by reducing the levels of ADMA (van Drongelen *et al.* 2011, Sasser *et al.* 2011, 2014). ROS production and NO-mediated relaxations are impaired in arteries of young, but not aged relaxin-deficient mice (Ng *et al.* 2015).

Exercise—Both *acute* and *chronic* exercise profoundly affect vascular reactivity in the coronary and skeletal muscle circulations, through upregulation of NO release and EDH-mediated responses resulting from increases in blood flow and thus shear stress (Whyte & Laughlin 2010, Padilla *et al.* 2011, Bender & Laughlin 2015, Bond *et al.* 2015). For example, exercise training prevents acute endothelial dysfunction whether due to the intake of a high-fat-containing meal (Bond *et al.* 2015) or induced by mental stress (Sales *et al.* 2014). Likewise, a single bout of lower limb interval exercise prevents endothelial dysfunction due to ischaemia–reperfusion injury (Seeger *et al.* 2015). Cardiac patients with lower physical activity levels exhibit endothelial dysfunction, to judge from reduced flow-mediated vasodilatations (Luk *et al.* 2012). In the rat, exercise training improves vasodilator responses to acetylcholine and ADP, as well as flow-mediated vasodilatation in aged animals, in part through increased release of NO (Spier *et al.* 2007, Mayhan *et al.* 2011, Xu *et al.* 2011, Jendzjowsky & DeLorey 2012), but this may not be the case in older humans (Kitzman *et al.* 2013). However, exercise training augments the NO contribution to cutaneous vasomotor responses to temperature changes in humans with non-alcoholic liver disease (Pugh *et al.* 2013).

Lipids—Normal *high-density lipoproteins* (HDL) associate with sphingosine 1-phosphate (S1P) and bind to S1P receptors of the endothelial cells, promoting/causing activation of eNOS and inducing the antioxidant enzyme heme oxygenase-1 (HO-1) (Tatematsu *et al.* 2013, Wu *et al.* 2013). Hence, the consensus is that high levels of normal HDL are protective against endothelial dysfunction and vascular disease. However, HDL of patients with coronary artery disease lose their protective properties and rather become inhibitors of eNOS (Besler *et al.* 2011, Gomaraschi *et al.* 2013, Tran-Dinh *et al.* 2013, Kratzer *et al.* 2014, Luscher *et al.* 2014). The chronic intake of *ω_3-unsaturated fatty acids* potentiates endothelium-dependent relaxations of coronary arteries to aggregating platelets and other stimuli and is anti-atherogenic (Shimokawa *et al.* 1987, 1988a, Shimokawa & Vanhoutte 1989a, Shepherd & Vanhoutte 1991, Von Schacky & Harris 2007, Sekikawa *et al.* 2008, Sena *et al.* 2008).

Natural products—Intake of a number of natural products favours endothelium-dependent relaxations. This holds true for the intake of *flavonoids* (Machha & Mustafa 2005, Machha *et al.* 2007, Xu *et al.* 2007, 2015, Liu *et al.* 2015b) and other *polyphenols* (in particular resveratrol), whether present in red wine (Stockley 1998, Leikert *et al.* 2002, Wallerath *et al.* 2002, Dell'Agli *et al.* 2004, da Luz & Coimbra 2004, Soares de Moura *et al.* 2004, Coimbra *et al.* 2005, Boban *et al.* 2006, Sarr *et al.* 2006, Das *et al.* 2007, Lefèvre *et al.* 2007, Aubin *et al.* 2008, Chan *et al.* 2008a,b, Csiszar *et al.* 2008, Lopez-Sepulveda *et al.* 2008, Scalera *et al.* 2009b, Dal-Ros *et al.* 2011, Idris Khodja *et al.* 2012, Li and Forstermann 2012), in green tea (Kuriyama *et al.* 2006, Alexopoulos *et al.* 2008, Jang *et al.* 2013), in grape juice (Anselm *et al.* 2007), in pomegranate juice (Nigris *et al.* 2006, 2007a,b) or in dark chocolate (Fisher *et al.* 2003, Engler *et al.* 2004, Grassi *et al.* 2005, Schroeter *et al.* 2006, Flammer *et al.* 2007, Taubert *et al.* 2007, Grassi *et al.* 2012). The protective effects of polyphenols against endothelial dysfunction, besides potentiation of EDH-mediated responses (Anselm *et al.* 2007, Idris Khodja *et al.* 2012, Xu *et al.* 2015),

involve mainly augmented production/bioavailability of NO in response to endothelium-dependent vasodilators (in particular insulin; Jang *et al.* 2013) resulting from: (i) antioxidant properties preventing the uncoupling of eNOS (Akar *et al.* 2011, Arrick *et al.* 2011, Dal-Ros *et al.* 2011, Lee *et al.* 2011a, Gordish & Beierwaltes 2014, Wang *et al.* 2014c); (ii) increased levels of BH4 (Carrizzo *et al.* 2013); (iii) calcium-independent phosphorylation of eNOS (Ramirez-Sanchez *et al.* 2011); (iv) activation of estrogen receptors (Yurdagul *et al.* 2014); (v) upregulation of AMPK and SIRT1 (Scalera *et al.* 2009b, Xu *et al.* 2011, Carrizzo *et al.* 2013, Warboys *et al.* 2014); and (vi) facilitation of the effects of endothelium-derived NO on the vascular smooth muscle cells (Xu *et al.* 2015). *Vanilloid* molecules, besides acting on TRPV1 expressed by perivascular nerves and releasing calcitonin gene-related peptide (CGRP), which act as a physiological antagonist of ET-1 (Félétou and Vanhoutte 2006c, Meens *et al.* 2009, see section Regulation of production and action of endothelin-1), also cause relaxations which are partly endothelium-dependent (with both NO and EDH contributing) and are due to the opening of endothelial TRPV4 channels (Peixoto-Neves *et al.* 2015), while their endothelium-independent vasodilator properties result from inhibition of L-type Ca^{2+} channels in vascular smooth muscle (Raffai *et al.* 2015).

Therapeutic agents—A number of available therapeutic agents can improve endothelium-dependent relaxations and alleviate endothelial dysfunction. For example: (i) adenosine triphosphate (ATP)-sensitive potassium (K_{ATP}) channel openers (Wang *et al.* 2011); (ii) α-glucosidase inhibitors (Sawada *et al.* 2014a,b); (iii) angiotensin-converting enzyme (ACE1) inhibitors (Tian *et al.* 2014); (iv) antihypertensive therapy (Lüscher *et al.* 1987c, Kang *et al.* 2011a); (v) DDP4 inhibitors (Liu *et al.* 2012b, Matsubara *et al.* 2012, Liu *et al.* 2014a, Salheen *et al.* 2015); (vi) fibrates (Glineur *et al.* 2013); (vii) AMPK activators (Rath *et al.* 2009, Sena *et al.* 2011); (viii) mineralocorticoid receptor (MR) antagonists (Schäfer *et al.* 2013); (ix) β_3-adrenoceptor antagonists (Khan *et al.* 2012, Zepeda *et al.* 2012); (x) renin inhibitors (Virdis *et al.* 2012); (xi) Rho-kinase inhibitors (Yao *et al.* 2013); or (xii) statins (Fig. 2) (Subramani *et al.* 2009, Datar *et al.* 2010, Fiore *et al.* 2011, Ghaffari *et al.* 2011, Zhang *et al.* 2012c, Kassan *et al.* 2013, Lee *et al.* 2013). In addition, the shortage of endogenous NO can obviously be bypassed by exogenous NO donors such as nitrite, by activators/stimulators of soluble guanylyl cyclase (sGC) or by phosphodiesterase inhibitors (Chester *et al.* 2011, Brown *et al.* 2014a, Goulopoulou *et al.* 2015).

Downregulation. Perturbed blood flow and high pressure—Abnormal blood flow patterns cause epigenomic DNA methylation and RNA processing changes which alter gene expressions, increase oxidative stress and precipitate senescence of the endothelial cells, thus blunting endothelium-dependent responsiveness and accelerating atherosclerosis development (Chiu & Chien 2011, Heo *et al.* 2011, De Verse *et al.* 2012, Davies *et al.* 2013, Dolan *et al.* 2013, Cybulsky & Marsden 2014, Warboys *et al.* 2014, Dunn *et al.* 2015, Stone *et al.* 2015 Wu *et al.* 2015). For example, senescence-associated β-galactosidase activity and the endothelial expression of p53 and of receptors for advanced glycation endproducts (RAGE) is elevated at sites of flow disturbance (De Verse *et al.* 2012, Warboys *et al.* 2014), while that of phosphatidic acid phosphatase type 2B (PPAP2B, an integral membrane protein that inactivates lysophosphatidic acid) is reduced (Wu *et al.* 2015). The accelerated senescence caused by perturbed flow can be alleviated by stimulation of SIRT1 (e.g. by the administration of resveratrol; Warboys *et al.* 2014). Prolonged exposure to high intraluminal pressures causes endothelial dysfunction (Huang *et al.* 1998, Millgard & Lind 1998, Paniagua *et al.* 2000, Vecchione *et al.* 2009), in part by increasing local angiotensin signalling and thus oxidative stress (Zhao *et al.* 2015).

MicroRNAs—A number of small, non-coding RNAs (miRs) affect vascular homeostasis (Bauersachs and Thum 2011, Shi & Fleming 2012, Zampetaki & Mayr 2012, Thum 2013, Boon & Dimmeler 2014, De Winther & Lutgens 2014, Frangogiannis 2014, Arunachalam *et al.* 2015). Of those, miR-155 appears to negatively modulate the expression/activity of eNOS and thus blunt endothelium-dependent relaxations (Sun *et al.* 2012).

Oxygen-derived free radicals—Several enzymes in the endothelial cells can produce superoxide anions (Fig. 7). They include nicotinamide adenine dinucleotide phosphate oxidase (NOX), xanthine oxidase, cyclooxygenases (COX) and eNOS itself, when it is uncoupled by lack of substrate (L-arginine) or shortage of BH4 (Kojda & Harrison 1999, Stuehr *et al.* 2001, Fleming *et al.* 2005, Tang *et al.* 2007, Zhang *et al.* 2011b, Virdis *et al.* 2013, Wu *et al.* 2014). However, physiological amounts of oxygen-derived free radicals, presumably of mitochondrial origin, can activate eNOS, increasing the production of NO and initiating/facilitating endothelium-dependent relaxations (Anselm *et al.* 2007, Feng *et al.* 2010, Rowlands *et al.* 2011, Bubolz *et al.* 2012). In addition, superoxide anions can be dismutated by superoxide dismutase

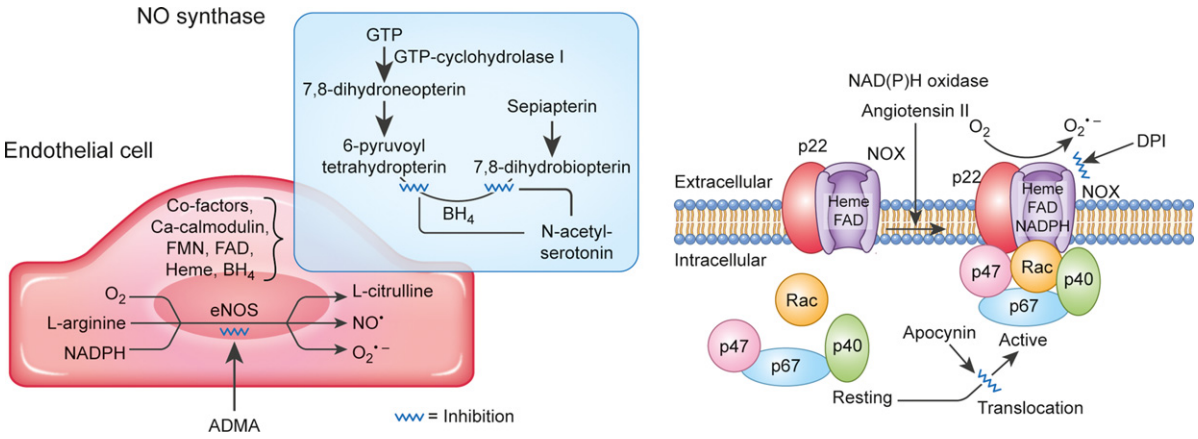

Figure 7 Two major contributors of reactive oxygen species in the vascular wall. *Left*: L-arginine-endothelial NOS (eNOS) pathway. The synthetic pathway of tetrahydrobiopterin (BH_4), an essential cofactor, is also shown and some of the most common inhibitors of NOS, analogues of L-arginine, are indicated. FMN, flavin mononucleotide; GTP, guanosine 5′-triphosphate. *Right*: activation of the NAD(P)H oxidase (NOX). Endothelial cells express NOX1, NOX2 ($gp91^{phox}$), NOX4 and NOX5 isoforms, whereas vascular smooth muscle cells express the NOX1, NOX4 and NOX5 and in resistance arteries NOX2 isoforms. Apocynin inhibits NOX by preventing translocation of cytosolic subunits and their association with the membrane located subunits, whereas diphenyleneiodonium (DPI), a flavoprotein inhibitor, is a non-specific inhibitor of NOX.

(SOD) to H_2O_2 which can act as an EDH factor and contribute to endothelium-dependent relaxations (Fig. 2; see section Endothelium-derived hydrogen peroxide). However, under pathophysiological conditions, superoxide anions scavenge NO avidly with the resulting formation of peroxynitrite (Gryglewski *et al.* 1986, Rubanyi & Vanhoutte 1986, Auch-Schwelk *et al.* 1992, Cosentino *et al.* 1994, Tschudi *et al.* 1996a, DeLano *et al.* 2006, Kagota *et al.* 2007, Miyagawa *et al.* 2007, Macarthur *et al.* 2008). This reduces considerably the bioavailability of NO (Kojda & Harrison 1999, Kietadisorn *et al.* 2012, Maron & Michel 2012, Montezano and Touyz 2012, Spescha *et al.* 2014). In addition, ROS cause *S*-glutathionylation of eNOS causing inactivity of the enzyme (Chen *et al.* 2010a, Dulce *et al.* 2011, Zhang *et al.* 2011b). Hence, increases in oxidative stress [initiated, e.g. by angiotensin II (Lee *et al.* 2011a, Yung *et al.* 2011, Sasser *et al.* 2014, Wang *et al.* 2014a,b), arsenic (Ellinsworth 2015), mercury (Furieri *et al.* 2011), 20-hydroxyeicosatetraenoic acid (20-HETE; Cheng *et al.* 2012), *β*-sitosterol (Yang *et al.* 2013), fibroblast growth factor 23 (Silswal *et al.* 2014), testosterone (Costa *et al.* 2015) or pollution (Wauters *et al.* 2013)] have been consistently associated with reduced endothelium-dependent relaxations. Such dysfunction can be curtailed by induction or overexpression of HO-1 (Cao *et al.* 2008, 2012, George *et al.* 2011). Likewise, antioxidants acutely improve endothelium-dependent relaxations *in vitro* and *in vivo* both in animals (Aubin *et al.* 2006, Liu *et al.* 2007, Costa *et al.* 2009, Dal-Ros *et al.* 2011, Idris Khodja *et al.* 2012, Lee *et al.* 2011b, Raffai *et al.* 2011, Yung *et al.* 2011, Wilcox 2012, Yang *et al.* 2013, Zhang *et al.* 2013, Gordish and Beierwaltes 2014, Wang *et al.* 2014a,c) and humans (Kanani *et al.* 1999, Taddei *et al.* 2001, Holowatz & Kenney 2007, Perampaladas *et al.* 2012, Schinzari *et al.* 2012, Virdis *et al.* 2012, Walker *et al.* 2012, Wray *et al.* 2012, Limberg *et al.* 2013, Fujii *et al.* 2014). Part of the improvement of endothelium-dependent-relaxations caused by HO-1 induction or exogenous antioxidants can be due to facilitation of EDH-mediated responses (Li *et al.* 2013a) or to the prevention of endothelium-dependent contractions (Tang *et al.* 2007, Tang & Vanhoutte 2009, Li *et al.* 2011; see section The major villains: endothelium-derived vasoconstrictor prostanoids). However, the therapeutic relevance of these findings in the animal is questionable as chronic treatment with antioxidants usually fails to improve endothelial function in people (Duffy *et al.* 2001, Pellegrini *et al.* 2004, Bjelakovic *et al.* 2007), with maybe the exception of the chronic administration of low doses of folic acid (Moat *et al.* 2006).

Asymmetric dimethylarginine—Asymmetric dimethyl arginine is an endogenous inhibitor of eNOS (Fig. 2); its production is accelerated by oxidative stress (Vallance and Leiper 2004, Wilcox 2012, Sasser *et al.* 2014) and proton pump inhibitors (Ghebremariam *et al.* 2013). Increased levels of ADMA result in blunted endothelium-dependent vasodilatations (Vallance and Leiper 2004, Antoniades *et al.* 2011, Wilcox 2012) and predispose to hypertension and car-

diovascular diseases (Knipp 2006, Palm *et al.* 2007, Teerlink *et al.* 2009, Antoniades *et al.* 2011, Wilcox 2012, Ghebremariam *et al.* 2013). The production of ADMA can be reduced by the induction of SIRT1 (Scalera *et al.* 2009b) and by serelaxin, possibly due to its antioxidant effects (Sasser *et al.* 2014). As regards the breakdown of ADMA, specific endothelial deletion of its major metabolizing enzyme dimethylarginine dimethylaminohydrolase (DDAH) does not affect responses to acetylcholine but impairs the angiogenic properties of the endothelial cells (Dowsett *et al.* 2015).

Salt intake—High-salt intake results in blunted endothelium-dependent relaxations (Lüscher *et al.* 1987b, Fiore *et al.* 2011, Hao *et al.* 2011, Pojoga *et al.* 2011, Beyer *et al.* 2014). This endothelial dysfunction can be prevented by antihypertensive therapy (Lüscher *et al.* 1987d), administration of statins (Fiore *et al.* 2011), missense mutation of extracellular SOD (Beyer *et al.* 2014), or by chronic activation of transient receptor potential vanilloid type 1 (TRPV1) channels with capsaicin (Hao *et al.* 2011). It can be exacerbated by striatin deficiency (Garza *et al.* 2015).

Aldosterone and epithelial sodium channels—The *acute* direct effects of aldosterone on isolated vascular cells are variable and concentration dependent. It can acutely augment NO-dependent relaxations through non-genomic processes (Uhrenholt *et al.* 2003, 2004, Skøtt *et al.* 2006, Nietlispach *et al.* 2007, Gros *et al.* 2013), although in cultured endothelial cells the hormone reduces NO release (Kirsch *et al.* 2013). The acute effects of aldosterone are complicated by its action on other cells in the vascular wall, for example the release of histamine from macrophages (Schjerning *et al.* 2013) or its interaction with estrogen receptors on the vascular smooth muscle cells (Gros *et al.* 2013). *Chronic* exposure to aldosterone has a detrimental effect on NO-dependent relaxations (Toda *et al.* 2013, Bauersachs *et al.* 2015), presumably by reducing the production of BH4 and increasing oxidative stress (Mitchell *et al.* 2004, Hashikabe *et al.* 2006, Nagata *et al.* 2006, Skøtt *et al.* 2006, Nietlispach *et al.* 2007, Sartorio *et al.* 2007) and thus augmenting the release of vasoconstrictor prostaglandins (Schäfer *et al.* 2013; see section The major villains: endothelium-derived vasoconstrictor prostanoids). In the pulmonary circulation, the reduced NO release may be due to the inactivation of ET_B receptors on the endothelial cells (Maron *et al.* 2012; see section Endothelin-1). One of the effects of aldosterone, presumably following intracellular sodium accumulation, is to augment the expression/presence of amiloride-sensitive epithelial sodium channels (ENaC) which results in greater 'stiffness' of the endothelial cells and a reduced NO release (Oberleithner 2005. Fels *et al.* 2010, Lang 2011, Jeggle *et al.* 2013, Paar *et al.* 2014). Potassium, by contrast, 'softens' endothelial cells and increases the release of NO (Rubanyi & Vanhoutte 1988, Oberleithner *et al.* 2009). The increased endothelial stiffness (and presumably the resulting reduced NO release) caused by aldosterone may help to understand the salutatory response to amiloride in adolescents with Liddle syndrome (Warnock 2013). However, vasodilator responses to amiloride may also reflect inhibition of the Na^+/H^+ exchanger (NHE). Thus, in animal arteries, amiloride *acutely* reduces contractions to phenylephrine and serotonin, in an eNOS- and flow-mediated manner (Cocks *et al.* 1988, Pérez *et al.* 2009). In rat and mice aortae, it causes endothelium-dependent relaxations, which are eNOS dependent, prevented by calmidazolium [a well established inhibitor of calcium-activated release of NO (Illiano *et al.* 1992, Nagao *et al.* 1992b)] and attributable to changes in intracellular pH modulating the cytosolic concentration of the eNOS-activating ion calcium (Sasahara *et al.* 2013).

Other hormones—Endothelium-dependent reactivity is reduced in Cushing patients with high *cortisol* levels (Chandran *et al.* 2011), while adrenalectomy augments the expression of eNOS (Li *et al.* 2007a). *Melatonin* inhibits the endothelial formation of NO (Silva *et al.* 2007). The beneficial effect of oestrogen therapy on G_i-dependent, endothelium-dependent relaxations is counteracted in animals and humans by the *chronic* administration of *progesterone* (Miller & Vanhoutte 1991, Miner *et al.* 2011). The opposing effects of oestrogens and progesterone could explain why delayed hormone replacement therapy has not always had the expected beneficial effect on the occurrence of cardiovascular events. Although *testosterone* can *acutely* cause dilatation *in vitro* [mediated by peroxynitrite (Puttabyatappa *et al.* 2013)] and appears to potentiate endothelium-dependent vasodilatation in post-menopausal women (Montalcini *et al.* 2007), *chronic* administration of the androgen hormone counteracts the improvement of endothelial function caused by oestrogen treatment in ovariectomized rats (Costa *et al.* 2015). Conversely, castration of male animals augments the vasodilator response to acetylcholine (Ajayi *et al.* 2004).

Ageing—Both in animals and in humans, increasing age reduces the ability of the endothelium to elicit endothelium-dependent vasodilatations *in vitro* and

in vivo (Moritoki *et al.* 1986, Hongo *et al.* 1988, Koga *et al.* 1988, Charpie *et al.* 1994, Kung & Lüscher 1995, Taddei *et al.* 1995a, 2001, Davidge *et al.* 1996, Cernadas *et al.* 1998, Yasuro *et al.* 1999, Heymes *et al.* 2000, Csiszar *et al.* 2002, 2007, Vanhoutte 2002, Subramanian & MacLeod 2003, Spier *et al.* 2007, Bulckaen *et al.* 2008, van Drongelen *et al.* 2011, Lesniewski *et al.* 2011, Wray *et al.* 2012, Chennupati *et al.* 2013, Feher *et al.* 2014, Gano *et al.* 2014, Trinity *et al.* 2014, Care *et al.* 2015, Ng *et al.* 2015). Besides diminished EDH-mediated relaxations (Chennupati *et al.* 2013, Care *et al.* 2015, Kong *et al.* 2015), reduced production of NO can contribute to the endothelial dysfunction associated with ageing. Such reductions in NO production have been attributed to: (i) increased activity of arginase, competing with eNOS for the common substrate arginine (Katusic 2007, Santhanam *et al.* 2007), (ii) augmented production of oxygen-derived free radicals reducing the bioavailability of NO (Tschudi *et al.* 1996a, Taddei *et al.* 2001, Csiszar *et al.* 2002, 2007, Ng *et al.* 2015) and (iii) reduced expression/presence of eNOS and reduced activity of the enzyme, possibly resulting from induction of nuclear factor kappa B (NFκB) (Challah *et al.* 1997, Cernadas *et al.* 1998, Chou *et al.* 1998, Csiszar *et al.* 2002, Lesniewski *et al.* 2011). Ageing also decreases the release of endothelium-derived vasodilator prostanoids, a deleterious effect which can be attenuated by the stimulation of SIRT1 with its activator SRT1720 (Gano *et al.* 2014). In addition, the expression of soluble guanylyl cyclase is reduced in aged vascular smooth muscle (Klöss *et al.* 2000). However, an important part of the endothelial dysfunction with ageing is due to the endothelial release of vasoconstrictor prostaglandins (see section Endothelin-1).

Smoking and environment—Active and passive *smoking* blunt endothelium-dependent vasodilatations. Besides a reduction in the release of endothelium-derived vasodilator prostanoids (Fujii *et al.* 2013), this appears to be due mainly to an action of nicotine causing a greater formation of ADMA and to an increased ROS production, both resulting in a lesser availability of NO (De Sousa *et al.* 2005, Michaud *et al.* 2006, Gamboa *et al.* 2007, Argacha *et al.* 2008, Celermajer & Ng 2008, Csiszar *et al.* 2008, Heiss *et al.* 2008, Lang *et al.* 2008, Frey *et al.* 2012, Fujii *et al.* 2013, 2014). Antenatal exposure to nicotine also results in blunting of endothelium-dependent relaxations after birth (Xiao *et al.* 2011). *Acute* and in particular *chronic* exposure to air *pollution* augment ROS production and decrease endothelium-dependent vasodilatations (Briet *et al.* 2007, Krishnan *et al.* 2012, Wauters *et al.* 2013).

Hypercholesterolaemia—Both in animals and in humans, hypercholesterolaemia in general and high levels of low-density lipoprotein cholesterol (LDL) in particular are accompanied by reduced endothelium-dependent relaxations/dilatations and the normalization of the cholesterol level with treatment restores the response (Shimokawa & Vanhoutte 1989a,b, Vanhoutte 1991, Trochu *et al.* 2003, Kaul *et al.* 2004, Landmesser *et al.* 2005, August *et al.* 2006, Fichtlscherer *et al.* 2006, Inoue & Node 2007, Aubin *et al.* 2008, Knight *et al.* 2008, Sena *et al.* 2008). Both the oxidized (oxyLDL; see section The weak link: regenerated endothelium) and the carbamylated forms of LDL are deleterious for NO-mediated responses (Boulanger *et al.* 1985, Cox & Cohen 1996, Speer *et al.* 2014). This is explained best by an increased oxidative stress leading to a reduced bioavailability of NO, an impairment of the turnover rate of eNOS, uncoupling of the enzyme, and an increased presence of ADMA (Bode-Bőger *et al.* 1996, Böger and Bode-Böger 2001, Böger *et al.* 2004, August *et al.* 2006, Palm *et al.* 2007, Speer *et al.* 2014).

Obesity—With few exceptions (Howitt *et al.* 2012), obese animals and humans exhibit reduced NO-mediated, endothelium-dependent relaxations/dilatations because of augmented ROS production and lesser phosphorylation of eNOS (Karagiannis *et al.* 2003, Van Guilder *et al.* 2006, 2008, Bouvet *et al.* 2007, Kagota *et al.* 2007, 2011, Mendizábal *et al.* 2011, Virdis *et al.* 2011, Beyer *et al.* 2012, Cao *et al.* 2012, Du *et al.* 2013, Liang *et al.* 2013, Limberg *et al.* 2013, Lynch *et al.* 2013a, Rinne *et al.* 2013, Schäfer *et al.* 2013, da Cunha *et al.* 2014, Azuma *et al.* 2015, Bradley *et al.* 2015). Deletion of Toll-like receptor 4 (TLR4) or adipocyte-specific overexpression of HO-1 attenuates the endothelial dysfunction induced by a high-fat diet (Cao *et al.* 2012, Liang *et al.* 2013). Contributing to the endothelial dysfunction, EDH-mediated relaxations are also impaired by diet-induced obesity (Haddock *et al.* 2011, Howitt *et al.* 2012), while the release of endothelium-derived vasoconstrictor prostanoids (see section The major villains: endothelium-derived vasoconstrictor prostanoids) is augmented (Sanchez *et al.* 2010, Mendizábal *et al.* 2011, Schäfer *et al.* 2013) and the ET-1 (see section Endothelin-1) system is upregulated (Weil *et al.* 2011, Virdis *et al.* 2013). Weight loss alone or exercise training improve endothelium-dependent responses (Watts *et al.* 2004, Focardi *et al.* 2007, Pierce *et al.* 2008, Ungvari *et al.* 2008). The favourable effect of caloric restriction on endothelium-dependent, NO-mediated relaxations can be attributed to the deacetylation and activation of eNOS by SIRT1 (Mattagajasingh *et al.* 2007).

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Adipokines—Certain adipokines, when released in exaggeration from inflamed adipose tissue, in particular PVAT, can curtail endothelium-dependent relaxations. *Chemerin* at low concentrations *acutely* induces endothelium-dependent relaxations but at high concentrations increases ROS production (and thus reduces NO bioavailability). It can also induce contraction of the vascular smooth muscle cells by directly increasing intracellular calcium levels, a response that is amplified by obesity (Watts *et al.* 2013, Neves *et al.* 2015). Likewise, although under physiological conditions, *leptin acutely* can induce endothelium-dependent relaxations *ex vivo* by enhancing NO (and EDH) production (Schinzari *et al.* 2013, Jamroz-Wiśniewska *et al.* 2014), but this effect is lost in patients with metabolic syndrome (Schinzari *et al.* 2013). While *chronic* exposure to leptin decreases NO bioavailability presumably by increasing oxidative stress which leads to depletion of endothelial NO and increases the levels of peroxynitrite (Korda *et al.* 2008). *In vivo* administration of exogenous *lipocalin-2* reduces endothelium-dependent relaxations to insulin *ex vivo* (Liu *et al.* 2012a; see section Endothelin-1). *Resistin* can also contribute to endothelial dysfunction by increasing oxidative stress and by activating the p38 and c-Jun NH2-terminal mitogen-activated protein kinase resulting in reduced eNOS expression and NO production (Chen *et al.* 2010b, Jamaluddin *et al.* 2012). *Visfatin* inhibits endothelium-dependent relaxations by stimulating the NOX and enhances ROS production (Vallejo *et al.* 2011, Xia *et al.* 2011). Besides interference with endothelial function, adipokines released from PVAT also reduce the ability of vascular smooth muscle to relax to adenosine (Noblet *et al.* 2015). A better understanding of the action of adipose tissue-derived factors in modulating vascular function may reveal a role for these molecules as potential diagnostic and prognostic cardiovascular markers as well as potential therapeutic target to reduce endothelial dysfunction.

Insulin resistance and metabolic syndrome—In obese animals and humans, the ensuing metabolic syndrome is characterized by insulin resistance resulting in blunted endothelium-dependent, NO-mediated relaxations due to reduced eNOS phosphorylation (Karki *et al.* 2015, Osto *et al.* 2015). The endothelial dysfunction and/or insulin resistance accompanying obesity, metabolic syndrome and diabetes has been attributed to: (i) increased ROS production by NOX leading to damages of the insulin receptors (Du *et al.* 2013); (ii) increased insulin receptor substrate (IRS)-1 phosphorylation at Ser307 (Nemoto *et al.* 2011); (iii) reduced production of epoxyeicosatrienoic acids (EETs) by cytochrome P450 (CYP 450) and augmented degradation of epoxy fatty acids by soluble epoxide hydrolase (sHE) (Abraham *et al.* 2014, Roche *et al.* 2015); (iv) upregulation of G protein-coupled receptor kinase 2 (GRK2) (Taguchi *et al.* 2014); (v) overexpression of protein kinase C-β (PKCβ) and induction of ET-1 expression in the endothelium (Lu *et al.* 2011b, Li *et al.* 2013b, Tabit *et al.* 2013); (vi) upregulation of transcription factor forkhead box O-1 (FOXO-1) (Karki *et al.* 2015); (vii) exaggerated activation of endothelial mineralocorticoid receptors (MR) by aldosterone (Schäfer *et al.* 2013); (viii) hypoadiponectinaemia resulting in downregulation of the adaptor protein that mediates adiponectin signalling (APPL1) (Xing *et al.* 2013); (ix) increased activity of ACE1 (Feher *et al.* 2013); (x) increased production of tumour necrosis factor-α (TNF-α) by PVAT(Virdis *et al.* 2011); (xi) exaggerated TLR4 activation (Liang *et al.* 2013); and (xii) downregulation in vascular smooth muscle cells of vasodilatory-stimulated phosphoprotein (VASP) (Cheng *et al.* 2014).

Homocysteinaemia—Increased levels of homocysteine impair eNOS-dependent relaxations/vasodilatations both *in vitro* and *in vivo*, presumably by upregulating sEH and increasing oxidative stress (Bellamy *et al.* 1998, Chambers *et al.* 1999, Kanani *et al.* 1999, Lang *et al.* 2000, Hanratty *et al.* 2001, Heil *et al.* 2004. Liu *et al.* 2007, Looft-Wilson *et al.* 2008, Sen *et al.* 2012, Zhang *et al.* 2012a). They also blunt EDH-mediated relaxations (Heil *et al.* 2004).

Uric acid—Depletion of uric acid, due to dysfunction of uric acid transporter 1 (URAT1), causes endothelial dysfunction in hypo-uricaemic patients, to judge from a reduced flow-mediated vasodilatation (Sugihara *et al.* 2015).

Hallmark of disease

Hypertension. Endothelium-dependent relaxations are reduced in isolated arteries from different animal models of hypertension (De Mey & Gray 1985, Lockette *et al.* 1986, Lüscher *et al.* 1987b,d, Hongo *et al.* 1988, Kung & Lüscher 1995, Vanhoutte & Boulanger 1995, Tschudi *et al.* 1996b, Vanhoutte 1996, Shimokawa & Vanhoutte 1997, Zhou *et al.* 1999, Johnson *et al.* 2005, Stec *et al.* 2008, Costa *et al.* 2009, Durand *et al.* 2010, Fiore *et al.* 2011, George *et al.* 2011, Hao *et al.* 2011, Lu *et al.* 2011b, Wang *et al.* 2011, El-Bassossy *et al.* 2012, Liu *et al.* 2012b, Zhang *et al.* 2013, 2014, Carrizo *et al.* 2013, Garcia *et al.* 2015, Hernanz *et al.* 2015). Likewise, the response to endothelium-dependent vasodilators is blunted in hypertensive humans (Taddei *et al.* 1995a,b, 1997b, 2001, Perticone *et al.* 2005, Levy *et al.* 2009, Virdis

et al. 2013). This blunting can be corrected by antihypertensive treatment both in animals and in people (Lüscher *et al.* 1987d, Hutri-Kahonen *et al.* 1997, Taddei *et al.* 1998, Benndorf *et al.* 2007, Naya *et al.* 2007, Kang *et al.* 2011a, Khan *et al.* 2012, Liu *et al.* 2012b, Zepeda *et al.* 2012) and probably reflects the premature ageing of the vasculature exposed chronically to the increased arterial blood pressure (Taddei *et al.* 1997b, 2001). In both hypertensive animals and humans, the reduced NO bioavailability in response to endothelium-dependent stimuli has been attributed to higher circulating levels of ADMA (Perticone *et al.* 2005, Sasser *et al.* 2014). The occurrence of dysfunction of the NO pathway accompanying an increased arterial pressure can be prevented/alleviated by a number of manoeuvres including: (i) activating NAD(P)H:quinone oxidoreductase (Kim *et al.* 2011); (ii) increasing the levels of BH4 (Kang *et al.* 2011b); (iii) inhibition of arginase (Johnson *et al.* 2005, El-Bassossy *et al.* 2012); (iv) activation of TRPV1 channels (Hao *et al.* 2011); (v) reduction in TLR4 signalling (Sollinger *et al.* 2014, Hernanz *et al.* 2015); (vi) missense mutation of extracellular SOD (Beyer *et al.* 2014); (vii) heterogenous deletion of GRK2 (Avendano *et al.* 2014); (viii) deletion of caveolin-1 (Rath *et al.* 2009, Pojoga *et al.* 2014); (ix) activation of ACE2 (Fraga-Silva *et al.* 2013); or (x) gene transfer of longevity-associated variant-BPIFB4 (Villa *et al.* 2015). Conversely, occurrence of endothelial dysfunction associated with hypertension can be precipitated by: (i) histone demethylase deficiency (Pojoga *et al.* 2011); (ii) deletion of peptidyl prolyl *cis-trans* isomerase (Pin1; Chiasson *et al.* 2011); (iii) deletion of collectrin (homolog of ACE2; Cechova *et al.* 2013); (iv) increase in the Rho-kinase activator RhoA (by administration of interleukin-17A; Nguyen *et al.* 2013); (v) deletion of α-CGRP (Smillie *et al.* 2014); (vi) deletion of small GTPase Ras-related protein 1 (Rap1b; Lakshmikanthan *et al.* 2014); (vii) endothelium-specific deletion of liver kinase B1 (LKB1); or (viii) administration of pentraxin 3 (PTX3; Carrizo *et al.* 2013).

Beyond NO—In the SHR, despite a lower expression of eNOS and soluble guanylyl cyclase in the arterial wall (Chou *et al.* 1998, Klöss *et al.* 2000, Michel *et al.* 2007), the blunting of endothelium-dependent relaxations/vasodilatations is due mainly to the concomitant release of endothelium-derived vasoconstrictor prostanoids (see section The major villains: endothelium-derived vasoconstrictor prostanoids) rather than to a reduced release of NO (Lüscher & Vanhoutte 1986, Lüscher *et al.* 1987c, Koga *et al.* 1988, Yasuro *et al.* 1999). Likewise in essential hypertensive humans, the decreased responsiveness to acetylcholine also can be corrected by inhibiting COXs (Taddei *et al.* 1997a, Virdis *et al.* 2013). In obese hypertensives, upregulation of the ET-1 system (see section Endothelin-1) also contributes to the endothelial dysfunction (Cardillo *et al.* 2004).

Diabetes. In arteries of diabetic animals and humans, the phosphorylation of eNOS is reduced and thus the NO-mediated endothelium-dependent relaxations/dilatations are impaired, presumably as a result of the chronic exposure to hyperglycaemia and the occurrence of insulin resistance (De Vriese *et al.* 2000, Vallejo *et al.* 2000, Cheng *et al.* 2001, Guzik *et al.* 2002, Inkster *et al.* 2002, Nassar *et al.* 2002, Pannirselvam *et al.* 2002, Kim *et al.* 2003, 2006, Shi *et al.* 2006, 2007a, Eringa *et al.* 2007, Goel *et al.* 2007, Machha *et al.* 2007, Obrosova *et al.* 2007, Cao *et al.* 2008, Schäfer *et al.* 2008, Akar *et al.* 2011, Kagota *et al.* 2011, Leo *et al.* 2011, Sena *et al.* 2011, Estrada *et al.* 2012, Li *et al.* 2012, 2013a, Patel *et al.* 2012, Romero *et al.* 2011, Yamaleyeva *et al.* 2012, Meijer *et al.* 2013, Rinne *et al.* 2013, Han *et al.* 2014, Kassan *et al.* 2014, Sawada *et al.* 2014a,b, van Sloten *et al.* 2014, Tian *et al.* 2014, Goulopoulou *et al.* 2015, Liu *et al.* 2015a). In the case of type 2 diabetes, a genetic predisposition to endothelial dysfunction may be involved (Iellamo *et al.* 2006). The mechanisms underlying the reduced NO-dependent dilatations in diabetes include: (i) reduced BH4 bioavailability and eNOS uncoupling (Guzik *et al.* 2002, Pannirselvam *et al.* 2002, Alp *et al.* 2003, Cai *et al.* 2005); (ii) increased activity of arginase, likely resulting from an augmented presence/activity of Rho-kinase (Ming *et al.* 2004, Ryoo *et al.* 2006, 2008, Katusic 2007, Lüscher & Steffel 2008, Romero *et al.* 2008, Vanhoutte 2008, Romero *et al.* 2011, El-Bassossy *et al.* 2012, Yao *et al.* 2013); (iii) elevated levels of ADMA (Lin *et al.* 2002, Xiong *et al.* 2003); (iv) augmented production of superoxide anions and thus scavenging of NO and increased presence of peroxynitrite (Cosentino *et al.* 1997, Mayhan & Patel 1998, Graier *et al.* 1999, Maejima *et al.* 2001, Inkster *et al.* 2002, Pannirselvam *et al.* 2002, Pacher & Szabo 2006, Duncan *et al.* 2007, Quijano *et al.* 2007, Gao *et al.* 2008, Lüscher & Steffel 2008, Schäfer *et al.* 2008, Leo *et al.* 2011, Sena *et al.* 2011, Huang *et al.* 2012, Cho *et al.* 2013); (v) NFκB activation (Liu *et al.* 2015b); (vi) suppression by FOXO1 of KLF2 (Lee *et al.* 2013); (vii) downregulation of stromal interaction molecule 1 (STIM1) and of sarcoplasmic endoplasmic reticulum protein 3 resulting in deficient endothelial intracellular calcium handling (Estrada *et al.* 2012); (viii) overexpression of RAGE receptors and quenching of NO by advanced glycosylation products (AGE; Bucala *et al.* 1991, Yin &

Xiong 2005, Gao *et al.* 2008, De Verse *et al.* 2012, Tian *et al.* 2014); (ix) reduced presence of apelin (Grisk 2007, Zhong *et al.* 2007); (x) altered metabolism in the endothelial cells (De Zeeuw *et al.* 2015); and (xi) reduced secretion of adiponectin by PVAT (Meijer *et al.* 2012). Endothelial diabetic dysfunction can be exacerbated by: (i) deletion of ACE2 (Patel *et al.* 2012); and (ii) p22*phox* expression causing dysfunction through an extracellular signal regulated kinase 1/2 (ERK1/2) and p38-mitogen-activated protein kinase-dependent mechanism (Kassan *et al.* 2014). Conversely, the blunting of NO-dependent responses in arteries of diabetic animals can be alleviated/prevented by the following manoeuvres: (i) knockin of eNOS with a single amino acid mutation at the S1176 phosphorylation site (Li *et al.* 2013a); (ii) induction of HO-1 (Cao *et al.* 2008, Tian *et al.* 2014); (iii) heterozygous knockout of Rho-kinase isoforms (ROCK 1 and ROCK 2; Yao *et al.* 2013); (iv) overexpression of STIM1 (Estrada *et al.* 2012); (v) KLF2 gene therapy (Lee *et al.* 2013); (vi) genetic deletion of sEH (Elmarakby *et al.* 2011); (vii) transgenic endothelium-specific overexpression of SIRT1 (Zhou *et al.* 2011); (viii) activation of ACE2 (Fraga-Silva *et al.* 2013); (ix) endothelium-selective overexpression of constitutively active AMPK (Li *et al.* 2012); (x) treatment with natural products such as boldine, 3,4-dihydroxyacetophenone or resveratrol (Akar *et al.* 2011, Lau *et al.* 2013, Liu *et al.* 2015a); (xi) administration of stable melanocortin analogs (Rinne *et al.* 2013); and (xii) treatment with existing therapeutic agents [e.g. losartan (Nemoto *et al.* 2011), metformin (Sena *et al.* 2011), miglitol (Sawada *et al.* 2014a,b) or ramipril (Tian *et al.* 2014)].

Beyond NO—Depending on the experimental model, EDH-mediated responses are blunted (Weston *et al.* 2008, Leo *et al.* 2011, Gokina *et al.* 2013, Schach *et al.* 2014) or unchanged (Kagota *et al.* 2011, Cho *et al.* 2013) in arteries of diabetic animals. Besides the reduced bioavailability of NO and altered EDH-like responses, the production of mainly endothelium-derived vasoconstrictor prostanoids (see section The major villains: endothelium-derived vasoconstrictor prostanoids), but possibly also that of adenosine tetraphosphate (Up4A) (Matsumoto *et al.* 2014) and ET-1 (see section Endothelin-1), contributes importantly to the endothelial dysfunction of diabetes. In addition, the responsiveness of vascular smooth muscle to endothelium-dependent vasodilators can be abnormal (Lu *et al.* 2005, 2011b, Lesniewski *et al.* 2008, Shi *et al.* 2008, Goulopoulou *et al.* 2015).

Coronary disease. Individuals at increased risk of coronary heart disease (CAD) are characterized by impaired peripheral dilatations (Ijzerman *et al.* 2003). Also in the coronary circulation, endothelial dysfunction is a characteristic of the disease, and the administration of intracoronary acetylcholine even can lead to the occurrence of vasospasm (Ludmer *et al.* 1986, Hodgson & Marshall 1989, Shimokawa & Vanhoutte 1997, Vanhoutte *et al.* 1997, Lavi *et al.* 2008, Ong *et al.* 2012, 2014, Ganz & Hsue 2013). Structural and functional modifications of HDL may explain the loss of their endothelial protective properties in patients with CAD (Besler *et al.* 2011, Kratzer *et al.* 2014, Hays *et al.* 2015). The coronary endothelial dysfunction in patients with CAD has also been associated with mitochondrial dysfunction resulting from too low levels of physical activity (Luk *et al.* 2012), with increased circulating ADMA levels (Antoniades *et al.* 2011) and with lower circulating levels of humanin (Widmer *et al.* 2013). Both in animals and humans, the presence of endothelial dysfunction predicts the severity of the outcome, in particular the occurrence of myocardial infarction and stroke (Suwaidi *et al.* 2000, Halcox *et al.* 2002, Kuvin & Karas 2003, Mancini 2004, Rossi *et al.* 2008).

Ischaemia–reperfusion injury. *Acutely* reperfused coronary arteries exhibit a reduced responsiveness to a variety of endothelium-dependent vasodilators (Pearson *et al.* 1990a, Huang *et al.* 2011). Also in intact humans, ischaemic injury reduces flow-mediated dilatations (McLaughlin *et al.* 2014). The impairment in NO bioavailability may be caused by increased ROS production leading to reduced activity of canonical transient receptor potential channel 3 (TRPC3) (Huang *et al.* 2011) or increased responsiveness of ET-1 receptors (Wackenfors *et al.* 2004, Martinez-Revelles *et al.* 2012). The impact of acute reperfusion injury on endothelial cells and eNOS can be mitigated by acetylcholine (He *et al.* 2015), nebivolol (Aragon *et al.* 2011), TRPC3 stimulation (Huang *et al.* 2011), sildenafil (McLaughlin *et al.* 2014), polyphenols (Shinmura *et al.* 2015, Yang *et al.* 2015) and caloric restriction (Shinmura *et al.* 2015). Ischaemia–reperfusion injury also has *chronic* endothelial consequences. Thus, 12 weeks later, reperfused coronary arteries exhibit impaired endothelium-dependent relaxations to aggregating platelets and platelet-derived compounds (ADP, serotonin and thrombin), but not to acetylcholine (Pearson *et al.* 1990b). This endothelial dysfunction resembles that observed in arteries with regenerated endothelium (Shimokawa *et al.* 1989, 1991, Chan *et al.* 2013) and suggests a chronic selective impairment of G_i-mediated responses.

Heart failure. Endothelium-dependent relaxations are reduced in coronary and peripheral arteries of animals and humans with ventricular hypertrophy and/or heart

failure presumably because of the increased oxidative stress resulting from underperfusion of the tissues leading to downregulation of eNOS and reduced bioavailability of NO (Kaiser *et al.* 1989, Treasure *et al.* 1990, Kubo *et al.* 1991, Katz *et al.* 1992, Zhao *et al.* 1995, Smith *et al.* 1996, Bauersachs *et al.* 1999, Indik *et al.* 2001, Nakamura *et al.* 2001, Landmesser *et al.* 2002, Malo *et al.* 2003, Trochu *et al.* 2003, Ferreiro *et al.* 2004, Widder *et al.* 2004, Lida *et al.* 2005, Gill *et al.* 2007, Rossi *et al.* 2008, Lam & Brutsaert 2012, Witman *et al.* 2012). An impairment of the vascular smooth muscle cells to relax contributes to the blunting of the endothelium-dependent responsiveness (Gill *et al.* 2007). Treatment with low doses of ouabain may improve NO release, as well as EDH-mediated responses, in arteries of rodents with heart failure (Siman *et al.* 2015). The degree of impairment of endothelium-dependent vasodilatations predicts the outcome in patients with chronic heart failure (Meyer *et al.* 2005). The peripheral endothelial dysfunction accompanying heart failure can be reversed, at least temporarily, by heart transplantation (Witman *et al.* 2012).

Pulmonary hypertension. *Chronic* hypoxia causing pulmonary hypertension is accompanied by reduced endothelium-dependent relaxations of pulmonary arteries, because of an overproduction of oxygen-derived free radicals leading to reduced activity of eNOS (resulting from a tighter coupling to caveolin-1) and a diminished bioavailability of NO (Fresquet *et al.* 2006, Jerkic *et al.* 2011, d'Uscio 2011, Shenoy *et al.* 2013, 2014, Kuriyama *et al.* 2014, Nozik-Grayck *et al.* 2014, Teichert-Kuliszewska *et al.* 2015). In the monocrotaline-induced form of the disease, a similar endothelial dysfunction caused by oxygen-derived free radicals occurs in the right ventricle (Sun & Ku 2006, Kajiya *et al.* 2007). The reduced NO release accompanying pulmonary hypertension can be accelerated by: (i) genetic deletion of bone morphogenetic protein receptors (Frank *et al.* 2008; see section The major villains: endothelium-derived vasoconstrictor prostanoids); (ii) heterozygous deletion of *Alk1* (coding for an endothelial-specific receptor for TGF-β; Jerkic *et al.* 2011); (iii) selective deletion of endothelial SOD (Nozik-Grayck *et al.* 2014); or (iv) reduction in the calcium-binding protein S100A1 (Teichert-Kuliszewska *et al.* 2015). It can be curtailed, for example, by the chronic administration of diminazene aceturate (DIZE, activator of ACE2; Shenoy *et al.* 2013) or by stimulation of the EPO system with genistein (Kuriyama *et al.* 2014).

Sleep apnoea. Intermittent hypoxia, as occurring with obstructive sleep apnoea, observed mainly in obese subjects, reduces endothelium-dependent responsiveness (Budhiraja *et al.* 2007, Butt *et al.* 2011, Azuma *et al.* 2015). The endothelial dysfunction caused by severe sleep apnoea is reversible (Butt *et al.* 2011, Azuma *et al.* 2015). To judge from work in the animal, it results from a reduction in NO release because of augmented oxidative stress and it does not involve EDH-mediated responses, but may be due in part to increased production of ET-1 (see section Endothelin-1; Capone *et al.* 2012, Crossland *et al.* 2013).

Inflammation. Inflammatory conditions can be accompanied by endothelial dysfunction in the human (Kharbanda *et al.* 2002, Higashi *et al.* 2008, Antoniades *et al.* 2011). To judge from studies in mice, the dysfunction is likely due to upregulation of NOX with increased ROS production causing a decreased NO bioavailability, and results from the NFκB induction by cytokines (Lesniewski *et al.* 2011, Karbach *et al.* 2014). In the animal, induction of *rheumatoid arthritis* causes blunting of endothelium-dependent relaxations due to elevated levels of monocyte chemotactic protein-1 (MCP-1) and BH4 deficiency, leading to exaggerated ROS production and eNOS uncoupling (Haruna *et al.* 2006, He *et al.* 2013, Totoson *et al.* 2014). The endothelial dysfunction can be ameliorated by statins (He *et al.* 2013). In patients with rheumatoid arthritis, flow-mediated dilatations are reduced, possibly because of augmented circulating levels of ADMA (Antoniades *et al.* 2011).

The weak link: regenerated endothelium

Endothelial cells form a monolayer mainly resulting from contact inhibition. After maturation of the body, they remain quiescent for many years before ageing and apoptotic programming initiate their turnover. However, the latter is accelerated by cardiovascular risk factors such as hypertension and diabetes. Eventually, the apoptotic cells die and are removed by the blood stream. They are replaced rapidly by regenerated endothelial cells. It is still uncertain what the exact contribution in this regeneration process is of neighbouring cells, freed of contact inhibition and circulating endothelial progenitor cells (Vanhoutte 1997, Hibbert *et al.* 2003, Sata 2003, Dimmeler & Zeiher 2004, Lamping 2007, Filipe *et al.* 2008, Zampetaki *et al.* 2008, Hagensen *et al.* 2012).

Regenerated endothelial cells are dysfunctional (Fig. 8). This conclusion is based on experiments performed on porcine coronary arteries (Shimokawa and Vanhoutte 1989a,b,c, Shimokawa *et al.* 1989, 1991, Eto *et al.* 2005, Chan *et al.* 2013). One month after *in vivo* balloon denudation of the endothelium of part of the artery, total relining of the endothelial surface

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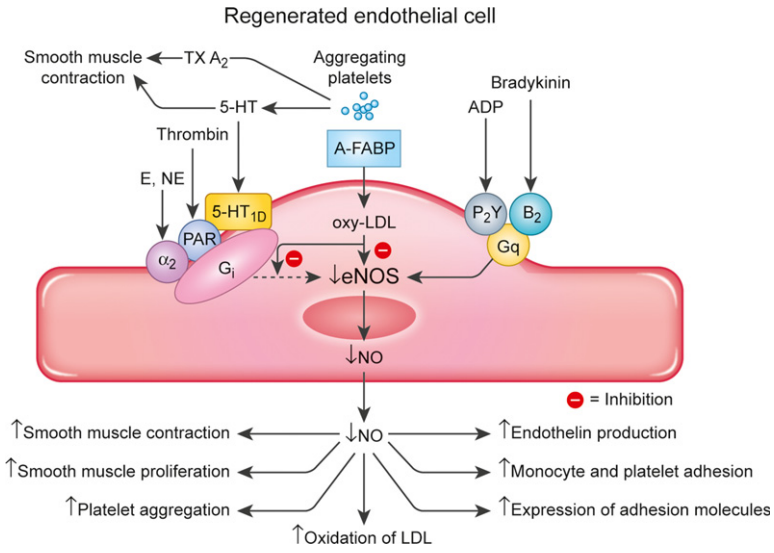

Figure 8 Effects of oxidized low-density lipoproteins (oxyLDL) in a regenerated endothelial cell, resulting in the reduced release of nitric oxide (NO). 5-HT, serotonin receptor; A-FABP, fatty acid binding protein-4; B, bradykinin receptor; P, purinoceptor; G, coupling proteins.

had occurred. Rings covered with regenerated endothelium exhibited a marked blunting of the relaxations to aggregating platelets, serotonin or thrombin and the remaining response was no longer inhibited by pertussis toxin. By contrast, relaxations evoked by ADP and bradykinin, which both depend on the G_q-signalling cascade, as well as those to the calcium ionophore A23187 were normal, illustrating the ability of the regenerated endothelial cells to produce NO. These findings implied a selective dysfunction of the G_i-dependent responses in regenerated endothelial cells. This selective dysfunction was reduced by the chronic intake of ω_3-unsaturated fatty acid, and exacerbated by a chronic hypercholesterolaemic diet which resulted in the occurrence of typical atherosclerotic lesions in the area of previous denudation. These observations prompt the conclusion that the selective dysfunction of regenerated endothelial cells is the first step allowing the atherosclerotic process.

To analyse the molecular mechanisms underlying the dysfunction of regenerated endothelial cells, primary cultures were derived from either regenerated or native endothelium (Borg-Capra *et al.* 1997, Fournet-Bourguignon *et al.* 2000, Kennedy *et al.* 2003, Lee *et al.* 2007). Primary cultures derived from regenerated endothelial cells had the appearance and markers of accelerated senescence, a reduced expression and activity of eNOS, a greater production of oxygen-derived free radicals (produced by the endothelial NOX), took up more modified LDL and generated more oxyLDL. By contrast, the presence of G_i proteins was comparable to that observed in primary cultures derived from the native endothelium. The genomic changes observed in cultures of regenerated endothelial cells were consistent with those phenotypic and functional changes. Increased extracellular concentrations of oxyLDL reduce the production of EDRF/NO and the endothelium-dependent relaxations to serotonin (Boulanger *et al.* 1985, Cox & Cohen 1996). Taken in conjunction, those observations prompt the conclusion that an augmented presence of oxyLDL is the cause of the selective loss in G_i protein-mediated responses of regenerated endothelial cells and thus of the inability to respond to serotonin and thrombin (Fig. 2). Obviously, this is not the only negative effect of oxygen-derived free radicals and oxyLDL which play a central role in the atherosclerotic process (Fig. 9) (Stocker and Keaney 2004, 2005, Li & Mehta 2005, August *et al.* 2006). Other factors include a direct inhibitory effect on the expression, reduced activation (dephosphorylation) and uncoupling of eNOS (Chu *et al.* 2005, Fleming *et al.* 2005, Brandes 2006, Heeba *et al.* 2007) and an enhanced activity of arginase, which competes with NO for the common substrate arginine (Fig. 6) (Ming *et al.* 2004, Brandes 2006, Ryoo *et al.* 2006, 2008, Katusic 2007, Romero *et al.* 2008, Vanhoutte 2008). In addition, a greater production of superoxide anions will reduce the bioavailability of NO and increase the levels of peroxynitrite (Kojda & Harrison 1999, Vanhoutte 2001, Fleming *et al.* 2005, Brandes 2006, Heeba *et al.* 2007).

Genomic factors and endogenous mediators, other than the increased presence of oxyLDL, may accelerate or contribute to the atherosclerotic process. These include: (i) emergence of fatty acid-binding proteins (Furuhashi *et al.* 2007, Lee *et al.* 2007, Furuhashi & Hotamisligil 2008, Hoo *et al.* 2008) and matrix-metalloproteinase 7 (MMP7; Lee *et al.* 2007); (ii) circulating chemokines (Ardigo *et al.* 2007); (iii) inhibition of the proteosome (Herrmann *et al.* 2007); (iv) presence of growth-related oncogene-α (Bechara *et al.* 2007); and (v) insufficiency of the paraoxonase-

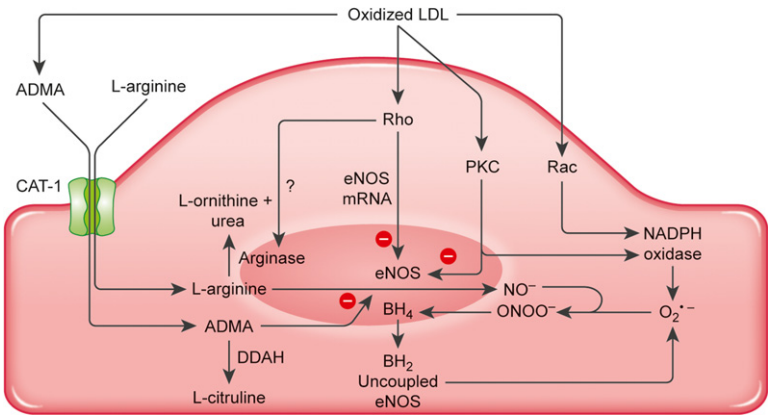

Figure 9 Mechanisms of oxyLDL-induced impairment of endothelial NO production. NO synthase (NOS) uses L-arginine to generate NO. NO production can be attenuated in the presence of oxyLDL by interfering with the supply of L-arginine to the enzyme through endogenous competitive inhibitors, in particular asymmetrical dimethyl-L-arginine (ADMA), as well as degradation of arginine through arginase. NOS expression and specific activity are decreased by oxyLDL through RhoA and PKC. NO bioavailability is reduced by an oxyLDL-mediated activation of NOX, which leads to superoxide anion (O_2^-) formation. This process facilitates the generation of peroxynitrite ($ONOO^-$), which subsequently oxidizes tetrahydrobiopterin (BH4), leading to NOS uncoupling. Uncoupled NOS itself produces O_2^-, further promoting the process of BH4 oxidation. Rho, member of the Rho protein family (either RhoA or Rac).

1 gene (Guns *et al.* 2008). Of those, the most relevant one may be adipocyte fatty acid-binding protein (A-FABP). Indeed, the mRNA of this protein is expressed in regenerated, but not native endothelial cells (Lee *et al.* 2007). This overexpression of A-FABP (as well as that of MMP7) requires the regeneration process to occur *in vivo*, as it is not observed in endothelial cells made senescent *in vitro* (Lee *et al.* 2010). In cultured human microvascular endothelial cells, lipid induction of A-FABP expression is associated with reduced phosphorylation of eNOS and NO production. These effects were reversed by the A-FABP inhibitor BMS309403 (Lee *et al.* 2011b). In the atherosclerosis-prone apolipoprotein E knockout ($ApoE^{-/-}$) mouse, the presence of A-FABP in the endothelium increases with age, in parallel with endothelial dysfunction involving G_i-mediated relaxations and the occurrence of atherosclerotic lesions (Lee *et al.* 2011b); chronic treatment with BMS309403 prevents the endothelial dysfunction (Lee *et al.* 2011b). Likewise, after endothelial regeneration following angioplasty in the coronary artery of the pig, chronic treatment with the A-FABP inhibitor prevents the selective reduction in endothelium-dependent responses to serotonin, as well as the intimal thickening following the procedure (Chan *et al.* 2013). A similar protection is exerted by chronic treatment with the antioxidant apocynin, comforting the interpretation that overexpression of A-FABP and the resulting increase in oxidative stress, and hence the augmented presence of oxyLDL in the endothelial cells plays a causal role in the genesis of endothelial dysfunction.

Whatever the cause is of their dysfunction, the endothelial cells cannot produce enough NO in response to platelets and thrombin and this NO deficiency permits the inflammatory reaction leading to atherosclerosis (Ross 1999, Aikawa & Libby 2004, Hansson 2005, Barton *et al.* 2007, Vanhoutte 2009b, Vanhoutte *et al.* 2009).

Endothelium-derived hydrogen peroxide

As mentioned in the Introduction, besides NO and prostacyclin, a number of other endothelial mediators and signals can cause endothelium-dependent, NO-independent hyperpolarization (EDH) and thus relaxation of the underlying vascular smooth muscle (Fig. 3) (Félétou & Vanhoutte 2006a,c, 2007, 2009, Shimokawa 2014). To discuss in depth these other endothelial signals is beyond the scope of this review, and the contribution of EDH to endothelial dysfunctions has been mentioned already. However, endothelium-derived H_2O_2 has gained prominence and will be discussed in more details in view of its emerging relevance for the coronary circulation.

Blood vessel size and relative contributions of endothelium-derived NO and H_2O_2

NO and endothelium-derived, hyperpolarizing H_2O_2, which under physiological conditions originates from NOS-derived superoxide anions dismutated by endothelial Cu,Zn-SOD (Morikawa *et al.* 2003, Takaki *et al.* 2008b), play diverse roles in modulating

vascular tone in a distinct blood vessel size-dependent manner. Indeed, NO is the dominant endothelium-dependent vasodilator in conduit arteries, while H_2O_2 is so in resistance vessels (Nagao *et al.* 1992a, Takaki *et al.* 2008a,b, Shimokawa 2014). The precise mechanism by which H_2O_2 relaxes the underlying vascular smooth muscle involves induction of protein kinase $G_{1\alpha}$ ($PKG_{1\alpha}$) dimerization and subsequent activation of large conductance calcium-activated potassium channels, leading to hyperpolarization and vasodilatation in murine resistance vessels as well as in human coronary arterioles (Burgoyne *et al.* 2007, Liu *et al.* 2011, Prysyazhna *et al.* 2012, Zhang *et al.* 2012b). Indeed, in mice, knockin of the mutant Cys42Ser $PKG_{1\alpha}$ yields vascular smooth muscle cells insensitive to H_2O_2-induced dimerization, due to the absence of a redox-sensitive sulphur; as a result, these animals exhibit markedly impaired EDH-mediated relaxations *ex vivo* and systemic hypertension *in vivo* (Prysyazhna *et al.* 2012). In addition, H_2O_2 also has potent vasodilator properties in coronary resistance vessels, so that impaired H_2O_2-mediated vasodilatations may lead to coronary microvascular dysfunction (Crea *et al.* 2014). Since coronary vascular resistance is predominantly determined by the pre-arterioles (more than 100 μm in diameter) and arterioles (<100 μm) where EDH-mediated responses become more important than NO-mediated relaxations for vascular tone (Crea *et al.* 2014), maintaining a proper blood vessel size-dependent contribution of NO vs. EDH appears essential for the treatment of coronary artery disease. Further mechanistic insight into the blood vessel size-dependent relative contributions of NO and H_2O_2 has been gained. Thus, compared with conduit arteries, in mouse resistance vessels, endothelial NO synthase is functionally suppressed by caveolin-1 and relaxation of their vascular smooth muscle cells to H_2O_2 is enhanced through a $PKG_{1\alpha}$-mediated mechanism (Burgoyne *et al.* 2012, Ohashi *et al.* 2012, Tsutsui *et al.* 2012). Furthermore, in mice, endothelial AMPK modulates EDH-mediated responses in resistance, but not in conduit arteries, contributing to the regulation of arterial blood pressure and coronary blood flow responses *in vivo* (Enkhjargal *et al.* 2014). Although three NO synthase isoforms including neural NO synthase (nNOS, NOS1), inducible NO synthase (iNOS, NOS2) and endothelial NOS (eNOS, NOS3) are expressed in the cardiovascular system, eNOS is the dominant NO synthase isoform in blood vessels (Forstermann & Li 2011). NO synthases have been known to generate superoxide anions from reductase domain under physiological conditions (Stuehr *et al.* 2001), where superoxide anions are converted to H_2O_2 to cause EDH-mediated responses. Since haeme reduction rate in eNOS is much slower than that in the other NO synthase isoforms, reductase domain-mediated superoxide generation is a significant alternative in eNOS (Stuehr *et al.* 2001). Based on these observations, eNOS may be the most important isoform in generating H_2O_2/EDH in the endothelium. Note that superoxide anions relevant to are not derived from pathologically uncoupled eNOS because the H_2O_2/EDH-mediated responses are resistant to NOS inhibitors and upregulation of eNOS cofactor tetrahydrobiopterin has no effects on the responses (Takaki *et al.* 2008a). This is the case at least in normal mesenteric arteries (Takaki *et al.* 2008a); however, in human coronary arterioles, other sources of superoxide anions have been proposed in H_2O_2-mediated vasodilatation as well; mitochondrial respiratory chain- and nicotinamide adenine dinucleotide phosphate (NADPH)-derived H_2O_2 is associated with flow-mediated dilation and bradykinin-induced vasodilatation respectively (Liu *et al.* 2003; Larsen *et al.* 2009).

Dual role of reactive oxygen species

Endothelium-derived H_2O_2 serves as an EDH factor to cause vasodiltation as described above, while it also induces endothelium-dependent vasoconstriction by COX-dependent release of thromboxane in rat mesenteric arteries (Garcia-Redondo *et al.* 2009) and causes vasoconstriction when hyperpolarization is compromised in perfused mouse mesenteric arteries (Lucchesi *et al.* 2005). The estimated concentration of endothelium-derived H_2O_2 as an EDH factor is in micromolar order (Matoba *et al.* 2003, Yada *et al.* 2006), which is much lower concentration than that observed in various pathological conditions (Schroder & Eaton 2008, Burgoyne *et al.* 2013). Although ROS, including H_2O_2, usually are regarded as primarily harmful, their protective role has attracted attention based on the accumulating evidence that endothelium-derived H_2O_2 participates in endothelium-dependent vasodilatation and contributes to vascular homeostasis at physiologically relevant low concentrations (Matoba *et al.* 2000, 2003, Morikawa *et al.* 2003, Shimokawa & Matoba 2004, Yada *et al.* 2006, Takaki *et al.* 2008b, Burgoyne *et al.* 2013, Godo *et al.* 2014, Satoh *et al.* 2014). As predicted (Vanhoutte 2001) following the initial description of the role of H_2O_2 in EDH (Matoba *et al.* 2000), the available evidence comforts the interpretation that H_2O_2 is a physiological signalling molecule serving as an EDH-inducing mediator especially in the microcirculation and thus modulates arterial blood pressure (Prysyazhna *et al.* 2012), metabolic coronary vasodilatation (Yada *et al.* 2007) and metabolic functions (Nakajima *et al.* 2012). Such physiological importance of H_2O_2 helps to understand

why, in the clinical setting, chronic antioxidant therapies do not improve mortality rate in patients or even worsen their prognosis (Bjelakovic *et al.* 2007), providing insight into the importance of ROS in maintaining homeostasis in humans.

Endothelium-dependent contractions

As stated in the Introduction, the endothelium cells can also initiate contractions of the underlying vascular smooth muscle cells (Fig. 3; De Mey & Vanhoutte 1982, 1983). Bioassay studies demonstrated that diffusible substances (EDCF) are responsible for these endothelium-dependent increases in vasomotor tone (Rubanyi & Vanhoutte 1985, Iqbal & Vanhoutte 1988, Yang *et al.* 2003). The delicate balance between endothelium-dependent relaxations and contractions maintains vascular homeostasis. Reduced productions of relaxing signals combined with an elevated secretion of EDCFs are the major characteristic of endothelial dysfunction (Wong & Vanhoutte 2010). Although endothelial cells can produce ET-1 (Yanagisawa *et al.* 1988, Yanagisawa & Masaki 1989, Schini & Vanhoutte 1991b, Vanhoutte 1993, Rubanyi & Polokoff 1994, Böhm & Pernow 2007, Kirkby *et al.* 2008; see section Endothelin-1) and other non-prostanoid vasoconstrictor substances (Cosentino *et al.* 1994, Dhein *et al.* 1997, Saifeddine *et al.* 1998, Jankowski *et al.* 2005), the available evidence strongly suggests that under most circumstances (except in the case of insulin resistance), vasoconstrictor prostaglandins produced by COX in the endothelium explain endothelium-dependent contractions (Vanhoutte *et al.* 2005, Félétou *et al.* 2011). The particular EDCF responsible for the endothelium-dependent contractions highly depends on the aetiology of the disease, the animal species studied or the specific vascular bed chosen for examination. Endothelium-dependent contractions are most prominent in arteries of aged, obese or diseased (e.g. hypertension, diabetes) animals and humans in which endothelial function is impaired (Barton 2010, Tang & Vanhoutte 2010, Vanhoutte 2013b). However, the occurrence of endothelium-dependent contractions also can occur in arteries and veins of young and healthy subjects (Wong *et al.* 2009).

The major villains: endothelium-derived vasoconstrictor prostanoids

Origin and action. Which cyclooxygenase isoform is responsible – the forever argument?—Endothelium-dependent, COX-dependent contractions (Fig. 10) to acetylcholine and other vasoactive substances (e.g. arachidonic acid, ATP, the calcium ionophore A23187) have been observed in blood vessels from different species (Furchgott & Vanhoutte 1989, Lüscher & Vanhoutte 1990, Kauser & Rubanyi 1995, Davidge & Zhang 1998, Kähönen *et al.* 1998, Derkach *et al.* 2000, Yang *et al.* 2003, Vanhoutte *et al.* 2005). Indeed, most endothelium-dependent contractions are prevented by non-selective inhibitors of COXs (Miller & Vanhoutte 1985, Lüscher & Vanhoutte 1986, Katusic *et al.* 1988), exemplifying the pivotal role of these enzymes in the phenomenon. Bioassay studies indicated that the vasoconstrictor prostanoids involved are produced by the endothelial COX, rather than that of the vascular smooth muscle (Yang *et al.* 2003). Two isoforms of the enzyme have been identified in blood vessels (Félétou *et al.* 2011). COX-1 is constitutively expressed, whereas COX-2 is highly inducible (Pritchard *et al.* 1994). As two iso-

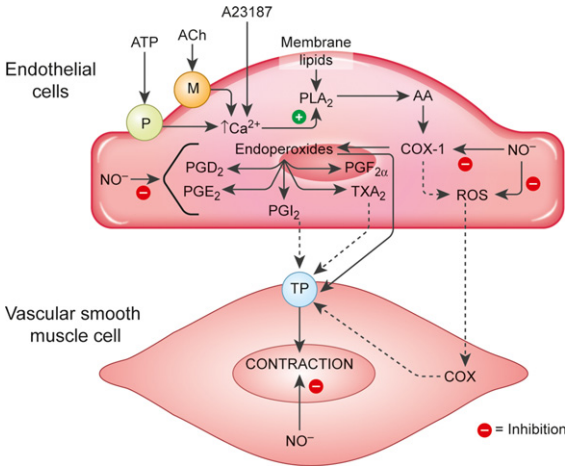

Figure 10 Endothelium-dependent contraction is likely to be comprised of two components: Generation of prostanoids and ROS. Each component depends on the activity of endothelial COX-1 and the stimulation of the TP receptors located on the smooth muscle to evoke contraction. In the SHR aorta, there is an increased expression of COX-1 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 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; X + XO, xanthine plus xanthine oxidase.

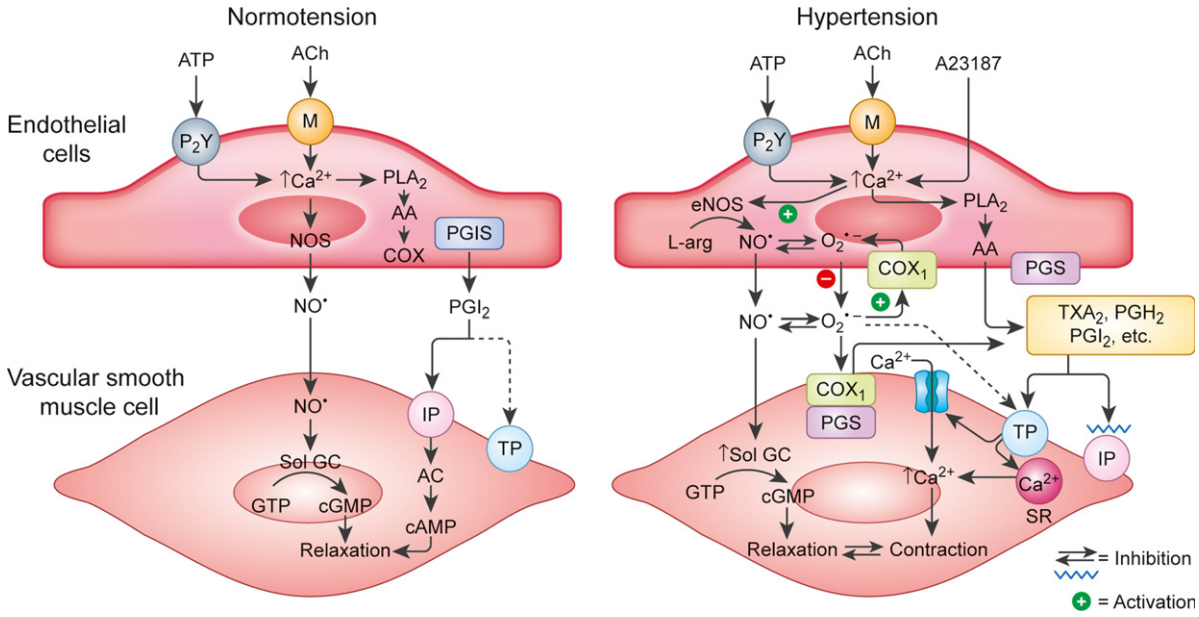

Figure 11 Endothelium-dependent effects of acetylcholine in the rat aorta. *Left*: endothelium-dependent relaxations in preparations of normotensive rats. *Right*: cyclooxygenase-dependent, endothelium-dependent contractions to acetylcholine in SHR aorta. PGI_2, prostacyclin; R, receptor; IP, prostacyclin receptor; TP, thromboxane receptor; PLA_2, phospholipase A_2; AA, arachidonic acid; COX1, cyclooxygenase 1; S-18886, antagonist of TP receptors; M, muscarinic receptor; PGIS, prostacyclin synthase; PGH2, endoperoxides; sGC, soluble guanylyl cyclase; AC, adenylyl cyclase; SR, sarcoplasmic reticulum; +, activation; −, inhibition; ?, unknown site of formation.

forms exist, the next obvious question was which of those two evokes endothelium-dependent contractions. Using preferential and selective inhibitors of the two isoforms of the enzyme, molecular biology experiments and studies with blood vessels of genetically modified mice concur to suggest that COX-1 is the major source of EDCF, as least in mouse arteries (Tang *et al.* 2005a, Zhou *et al.* 2013) and in the aorta of SHR and diabetic rats (Ge *et al.* 1995, Traupe *et al.* 2002b, Ospina *et al.* 2003, Wang *et al.* 2003, Yang *et al.* 2003, Gluais *et al.* 2006, Virdis *et al.* 2013). In these arteries, the expression of COX-1 is upregulated, which facilitates the enhanced production of vasoconstrictor prostanoids in the endothelium that diffuse to contract the underlying vascular smooth muscle (Félétou *et al.* 2009, Tang & Vanhoutte 2009). The consequences of the upregulation of COX-1 expression in these arteries can be reversed by preferential selective inhibitors of this isoform. However, under pathological conditions, including hypertension, diabetes and obesity, the expression of COX-2 in arteries is often upregulated (Pritchard *et al.* 1994, Camacho *et al.* 1998, Zerrouk *et al.* 1998, Garcia-Cohen *et al.* 2000, Ikeda *et al.* 2008, Shi & Vanhoutte 2008). Additionally, the expression of COX-2 increases with ageing and with augmented shear stress resulting from exaggerated pulsatile flow (Topper *et al.* 1996, Hendrickson *et al.* 1999, Wong *et al.* 2009). COX-2 can also be expressed constitutively, as is the case in the endothelium of the rat pulmonary and human renal arteries and in cultured endothelial cells (Baber *et al.* 2003, Therland *et al.* 2004). In situations where endothelial COX-2 is present/induced, the prostanoids generated by this isoform can evoke endothelium-dependent contractions (Camacho *et al.* 1998, Zerrouk *et al.* 1998, Garcia-Cohen *et al.* 2000, Blanco-Rivero *et al.* 2005, Hirao *et al.* 2008, Shi & Vanhoutte 2008). For example, in carotid and renal arteries of the SHR, the exaggerated endothelium-dependent contractions in these arteries are selectively inhibited by COX-2 inhibitors, indicating that the endothelium-dependent contractions in these arteries are mediated by COX-2 upregulation (Wong *et al.* 2009, Liu *et al.* 2014b, 2015b). In human essential hypertension, COX-2 is overexpressed and appears to be the major isoform responsible for endothelial dysfunction (Virdis *et al.* 2013). Hence, both COX isoforms can evoke endothelium-dependent contractions (Vanhoutte 2013a). The contribution of the specific COX isoforms to such contraction highly depends on their relative expression in the particular artery of interest.

Calcium, the trigger for release—Although the release of EDCF can be tonic (Iwatani *et al.* 2008) or elicited by sudden stretch (Katusic *et al.* 1987), it usually is

initiated by vasoactive mediators acting at the cell membrane, including acetylcholine [activating endothelial M3-muscarinic receptors (Boulanger *et al.* 1994)] or adenosine di- and triphosphate [activating purinoceptors (Koga *et al.* 1989, Mombouli & Vanhoutte 1993)]. These mediators activate calcium-independent phospholipase A_2 ($iPLA_2$) that produces lysophospholipids, which in turn open store-operated calcium channels, permitting the influx of extracellular calcium which subsequently activates calcium-dependent phospholipase A_2 ($cPLA_2$) (Wong *et al.* 2010a), which then makes arachidonic acid available for endothelial COX, setting in motion the release of EDCF. Indeed, the store-operated calcium channel inhibitor, SKF-96565, prevented endothelium-dependent contraction to acetylcholine (Wong *et al.* 2010a). Exposure of arteries to 2-aminothoxydiphenyl borate, a non-selective cation channel blocker, diminished endothelium-dependent contractions (Wong *et al.* 2009). Fitting with this notion, endothelium-dependent contractions are less prominent in bathing solution containing low extracellular calcium and are absent when extracellular calcium ions are removed (Wong *et al.* 2009). Reintroduction of calcium to the bathing solution restores endothelium-dependent contraction to acetylcholine (Wong *et al.* 2009). These experiments permitted the conclusion that an increase in intracellular calcium concentration is pivotal for the initiation of endothelium-dependent contractions (Katusic *et al.* 1988, Okon *et al.* 2002, Gluais *et al.* 2006, Shi *et al.* 2007a,b, 2008, Tang *et al.* 2007, Wong *et al.* 2008, 2010a). For these reasons, the calcium ionophore A23187, a compound that allows calcium ions to cross membranes thereby increasing intracellular calcium levels, is often used experimentally to induce endothelium-dependent contractions (Yang *et al.* 2004, Shi *et al.* 2007a, Tang *et al.* 2007, Qu *et al.* 2010).

EDCF candidates: when prostacyclin turns bad—COX transforms arachidonic acid into endoperoxides which are released during endothelium-dependent contractions. Endoperoxides *per se* can activate vascular smooth muscle and thus are considered as plausible EDCF candidates (Ito *et al.* 1991, Asano *et al.* 1994, Ge *et al.* 1995, Vanhoutte *et al.* 2005, Hirao *et al.* 2008). Endoperoxides diffusing from the endothelium could be processed in the medial smooth muscle into prostacyclin and then activate TP receptors, contributing to the vasoconstrictor activity of endothelial COX-derived metabolites (Zhou *et al.* 2013). However, the majority of endoperoxides are converted in the endothelial cells into prostacyclin, thromboxane A_2, prostaglandin D_2, prostaglandin E_2 and/or prostaglandin $F_{2\alpha}$ by their selective synthases (Bos *et al.* 2004). The expression of the prostacyclin synthase gene is the most abundant in endothelial cells from mouse and rat aorta (Gluais *et al.* 2005, Tang & Vanhoutte 2008b). During endothelium-dependent contractions to acetylcholine, the release of prostacyclin outweighs that of other prostaglandins (Gluais *et al.* 2005). In arteries where endothelium-dependent contractions to the muscarinic agonist are prominent, prostacyclin does not evoke relaxation of the vascular smooth muscle but instead causes contraction (Rapoport & Williams 1996, Gluais *et al.* 2005). Thus, it seems logical to conclude that endoperoxides and prostacyclin are the main mediators of these responses, at least for those evoked by acetylcholine (Ge *et al.* 1995, Blanco-Rivero *et al.* 2005, Gluais *et al.* 2005) in rat and mouse aortae. In situations where endothelium-dependent contractions are mediated by other agonists (including ADP, A23187, ET-1, thrombin and nicotine), thromboxane A_2 also contributes (Katusic *et al.* 1988, Shirahase *et al.* 1988, Auch-Schwelk & Vanhoutte 1992, Taddei & Vanhoutte 1993, Derkach *et al.* 2000, Gluais *et al.* 2006, 2007). For instance, thromboxane A_2 is a significant EDCF in the canine basilar artery and in the SHR aorta (Katusic *et al.* 1988, Auch-Schwelk & Vanhoutte 1992, Gluais *et al.* 2006, 2007). In addition, studies in human renal arteries confirm that endothelial COX-2 catalyses the formation of prostaglandin $F_{2\alpha}$, which is the predominant EDCF in this preparation (Wong *et al.* 2009). Likewise, prostaglandin $F_{2\alpha}$ is the main EDCF in renal arteries of rats with hypertension induced by renal artery stenosis (Tian *et al.* 2012). Of interest, COX-2-derived prostaglandin $F_{2\alpha}$ is the main EDCF in the aorta of young and healthy hamsters and the contribution of this prostaglandin becomes increasingly significant in causing endothelium-dependent contraction upon ageing (Wong *et al.* 2009, 2010c). Therefore, the precise nature of the EDCFs varies among species and vascular beds. The relative expression of respective prostaglandin synthases, the extent of oxidative stress and the vasoactive mediators involved are among some of the factors which can influence the precise chemical nature of the prostanoid(s) triggering endothelium-dependent contractions.

TP receptors, the effectors—COX-dependent, endothelium-dependent contractions are inhibited by antagonists of TP receptors (Tesfamariam *et al.* 1989, Auch-Schwelk *et al.* 1990, Kato *et al.* 1990, Mayhan 1992, Yang *et al.* 2002, 2003, Zhou *et al.* 2005). The TP receptors involved are those of the vascular smooth muscle which initiate the contractile response (Yang *et al.* 2003). Changes in extracellular pH affect the responsiveness of the TP receptors. Extracellular

acidosis prevents and alkalosis augments endothelium-dependent contractions in mouse arteries (Baretella *et al.* 2014), without affecting acetylcholine-induced, eNOS-dependent relaxations. The release of vasoconstrictor prostanoids was not altered by extracellular changes in pH, demonstrating modulation of the responsiveness of TP receptors on the vascular smooth muscle cells to the unchanged release of EDCF (Baretella *et al.* 2014). Of note, intracellular acidification of endothelial cells and smooth muscle cells inhibits eNOS activity and endothelium-dependent relaxation (Boedtkjer *et al.* 2011).

Modulation of EDCF-mediated responses. NO, the gatekeeper—Inhibitors of NO synthases cause an immediate potentiation of EDCF-mediated responses (Auch-Schwelk *et al.* 1992, Yang *et al.* 2002). This is why experimentally, it is common to add NO synthase inhibitors, such as Nω-Nitro-L-arginine methyl ester (L-NAME) to the preparations to inhibit NO production and enhance the amplitude of endothelium-dependent contractions. However, the effect of NO is not only acute, but previous exposure to endogenous NO released from the endothelial cells or to exogenous NO donors causes a long-term inhibition of endothelium-dependent contractions (Tang *et al.* 2005b). These observations imply that any condition resulting in a lesser bioavailability of NO will favour the occurrence of EDCF-mediated contractions and the precipitation of endothelial dysfunction (Félétou *et al.* 2008, 2011, Vanhoutte *et al.* 2009). *Vice versa*, EDCF can curtail relaxation mediated by EDRFs (e.g. NO, prostacyclin, and EDH) by promoting increases in blood vessel tone. In particular, thromboxane A_2 can modulate several signalling components central to EDH – including direct modulation of the hyperpolarization (of both endothelial and vascular smooth muscle cells) mediated by the opening of calcium-activated potassium channels, as well as the subsequent conduction of hyperpolarization through gap junctions (Ellinsworth *et al.* 2014). The crosstalks between EDRFs and EDCFs provide another level of control and ensue fine regulation of vascular tone.

Impact of oxidative stress—Increased ROS levels play a major regulatory role on vascular tone. Superoxide anions effectively inactivate EDRF-NO, thus reducing its bioavailability and favouring the occurrence of endothelium-dependent contractions (Gryglewski *et al.* 1986, Rubanyi & Vanhoutte 1986, Auch-Schwelk *et al.* 1992, Cosentino *et al.* 1994, Tschudi *et al.* 1996b, Touyz & Schiffrin 2004, DeLano *et al.* 2006, Miyagawa *et al.* 2007, Macarthur *et al.* 2008). In particular, under conditions of high oxidative stress (e.g. in the presence of high glucose), peroxynitrite is formed by the reaction of NO with superoxide anions, which leads to tyrosine nitration and subsequent inactivation of prostacyclin synthase (Zou *et al.* 2002). This may result in a compensatory production of prostaglandin E_2 and $F_{2\alpha}$ which then become increasingly responsible for endothelium-dependent contractions (Zou *et al.* 1999, Bachschmid *et al.* 2003, Gluais *et al.* 2005). ROS have been identified as EDCF *per se* in the canine basilar artery (Katusic & Vanhoutte 1989) and in rat renal artery (Gao & Lee 2005). Free radicals can presumably diffuse to the underlying vascular smooth muscle and activate COX in the vascular smooth muscle cells (Auch-Schwelk *et al.* 1989, Katusic & Vanhoutte 1989, Garcia-Cohen *et al.* 2000, Yang *et al.* 2002, 2003, Wang *et al.* 2003, Shi & Vanhoutte 2008), thereby producing prostanoids that in turn activate TP receptors. The oxygen-derived free radicals may reach the vascular smooth muscle cells through the shielded channels constituted by the myo-endothelial gap junctions (Tang & Vanhoutte 2008a). ROS can amplify rather than directly induce endothelium-dependent contractions. This interpretation is based on the observations that in a number of arteries, cell-permeable ROS scavengers, such as tempol, variably depress endothelium-dependent contractions (Auch-Schwelk *et al.* 1989, Yang *et al.* 2002, 2003, Tang & Vanhoutte 2008a). In arteries such as the mouse aorta, the renal arteries of hypertensive rats, an increased production of ROS stimulates endothelial COX-2 to release more ROS and prostanoids which in turn activate TP receptors to cause contraction of the vascular smooth muscle cells (Tang *et al.* 2007, Tian *et al.* 2012). Therefore, ROS serve as a trigger/amplifier for the release of EDCF leading to greater amplitude of the resulting endothelium-dependent contractions.

At the initiation of the endothelium-dependent contractions that they evoke, acetylcholine and A23187 cause a burst of endothelial free radical production (Tang *et al.* 2007). Since the burst is prevented by indomethacin, COX appears to be one of the main sources of superoxide anions in endothelial cells (Tang *et al.* 2007). This is apparently also the case in the human forearm vasculature, where both COX inhibitors and antioxidant curtail endothelial dysfunction (Virdis *et al.* 2013). Various other enzymes generate intracellular ROS, including xanthine oxidase, uncoupled NO synthases, mitochondrial oxidases, lysyl oxidase and NOX. The endoplasmic reticulum (*ER*) stress-induced unfolded protein response (UPR) also produces ROS (Lenna *et al.* 2014). The NO scavenging properties of, and the facilitation of endothelium-dependent contractions exerted by ROS help to understand why substances that decrease oxidative stress confer vascular protec-

tion. For instance, berberine (a botanical alkaloid purified from *Coptidis* rhizomes) inhibits ER stress by activating AMPK (Liu *et al.* 2015b). In SHR carotid arteries, berberine inhibits the expression of a number ER stress-related protein (including eukaryotic translation initiation factor 2A, X-box-binding protein 1 and activating transcription factor-2 and -6) and reduces ROS production leading to COX-2 downregulation (Liu *et al.* 2015b). The natural antioxidant defence represented by the uncoupling proteins can scavenge mitochondrial ROS and lower oxidative stress (Liu *et al.* 2014b). For example, sitagliptin [a selective DPP4 inhibitor which upregulates the expression of uncoupling protein 2 (UCP2)] reduces COX-2 expression by lowering oxidative stress in SHR arteries (Liu *et al.* 2014b). Similar observations in aortae of angiotensin II-infused mice indicate that sitagliptin normalizes ROS overproduction and attenuates endothelium-dependent contractions, an effect absent in UCP2 knockout mice (Liu *et al.* 2014b). Fitting with this notion, UCP2 expression is reduced in the renal arteries of SHR compared with Wistar-Kyoto rat (WKY), and overexpression of UCP2 inhibits endothelium-dependent contractions in the former (Liu *et al.* 2014b). Hence, the mitochondrial UCP2 negatively regulates intracellular ROS production and prevents the occurrence of endothelium-dependent contractions. This interpretation is consistent with the observation that endothelium-dependent contractions are attenuated by mitochondrial ROS scavengers such as coenzyme Q10 and idebenone (Liu *et al.* 2014b). HO-1 is another negative regulator of intracellular ROS, which reduces the expression and activity of vascular COX in diabetic rats and hence influences the production of vasoconstrictor prostanoids (Wang *et al.* 2014d). Compounds such as hemin and tricarbonyldichlororuthenium (II) dimer, which induce the expression of HO-1, or release the HO-1 product carbon monoxide, respectively, suppress ROS production, inhibit COX-2 upregulation and impair endothelium-dependent contractions in the SHR aorta (Li *et al.* 2011, Wang *et al.* 2014d). Taken in conjunction, ROS amplify endothelium-dependent contractions by inactivating NO, acting as an EDCF *per se*, and/or by upregulating the expression and activity of COXs.

Oestrogens and gender—In arteries of ovariectomized animals, chronic treatment with oestrogens reduces the augmented production of vasoconstrictor prostanoids by endothelial COX1 and reduces the augmented responsiveness of the TP receptors of the vascular smooth muscle cells (Davidge & Zhang 1998, Dantas *et al.* 1999, Ospina *et al.* 2003). Oestrogens also reduce acutely EDCF-mediated responses in an NO-independent way (Zhang & Kosaka 2002). The production of endothelium-derived prostanoids is larger in arteries from male than female animals (Kauser & Rubanyi 1995, Kähönen *et al.* 1998). It is tempting to assume that the lesser occurrence of cardiovascular disease in women prior to menopause is related in part to the braking effect of oestrogens on EDCF-mediated responses.

Other hormones—Chronic treatment with *thyroid hormone* reduces the release of endothelium-derived vasoconstrictor prostanoids in arteries of diabetic animals (Cai *et al.* 2015). *GLP1* curtails endothelium-dependent contractions (Liu *et al.* 2014b).

Age—Endothelium-dependent contractions become more prominent with ageing (Koga *et al.* 1988, 1989, Iwama *et al.* 1992, Kung & Lüscher 1995, Heymes *et al.* 2000, Abeywardena *et al.* 2002, Matsumoto *et al.* 2007). The age dependency of the response is explained best by an increased oxidative stress resulting in the upregulation of COXs (Ge *et al.* 1995, Heymes *et al.* 2000, Matsumoto *et al.* 2007, Shi *et al.* 2008, Tang & Vanhoutte 2008b). In addition, the expression of the prostacyclin synthase gene augments with age (Numaguchi *et al.* 1999). Prostacyclin no longer evokes relaxations in arteries from ageing animals (Levy 1980, Rapoport & Williams 1996, Gluais *et al.* 2005). Inhibitors of COX, given *in vivo* or *in vitro*, prevent or revert, respectively, the blunting of endothelium-dependent relaxations due to ageing (Koga *et al.* 1988, 1989, Davidge *et al.* 1996, Wang *et al.* 2003, Bulckaen *et al.* 2008, Virdis *et al.* 2013). Indomethacin augments the relaxations to acetylcholine in isolated arteries of older patients as well as the vasodilator response to the muscarinic agonist in the forearm of ageing people, suggesting that the importance of EDCF-mediated responses also increases with age in the human (Lüscher *et al.* 1987a, Taddei *et al.* 1995a,b, 1997a,b).

Vitamin D—Epidemiologic studies imply that a low vitamin D status is associated with impaired vascular function (Tare *et al.* 2011, Ott *et al.* 2013, Hashemi *et al.* 2015; see section Nitric oxide). Chronic treatment with calcitriol, the active form of vitamin D, reduces the amplitude of *ex vivo* endothelium-dependent contractions in arteries of hypertensive rats (Wong *et al.* 2008, 2010b, Dong *et al.* 2012). The calcitriol-induced protection involves VDR activation leading to the downregulation of the expression/presence of AT1 receptors and NOX subunits which in turn prevents ROS overproduction (Wong *et al.* 2008, 2010b, Dong *et al.* 2012). This modulation is also

accompanied by reduced COX-1 mRNA expression and protein presence, as well as by a lowered arterial blood pressure (Wong *et al.* 2008, 2010b, Dong *et al.* 2012). These changes have been confirmed in human renal arteries but, however, are not observed in normotensive animals (Wong *et al.* 2008, 2010b, Dong *et al.* 2012). Thus, hypertensive individuals with vitamin D deficiency may be more prone to endothelial dysfunction characterized by increased occurence of endothelium-dependent contractions/constrictions.

Bone morphogenetic protein 4—This protein, originally thought to participate mainly in embryonic development and bone and cartilage formation, exerts a wide range of pathophysiological activities, including the initiation and maintenance of endothelial dysfunction (Wong *et al.* 2010c). Diseases characterized by enhanced ROS production (e.g. cancers and hypertension) are accompanied by augmented cellular and circulating levels of bone morphogenetic protein 4 (BMP4; Miriyala *et al.* 2006, Kallioniemi 2012, Guo & Dong 2014). In the mouse, the infusion of exogenous BMP4 impairs acetylcholine-induced relaxations *ex vivo* through stimulation of NOX, leading to ROS-dependent overexpression of COX-2 in endothelial cells and exaggerating the occurrence of endothelium-dependent contractions (Wong *et al.* 2010c). Such BMP4-induced endothelial dysfunction is absent in COX-2 deficient mice (Wong *et al.* 2010c), supporting the interpretation that the inducible COX isoform is responsible for the abnormal endothelium-dependent relaxations and the predominance of EDCF-mediated responses. Silencing of BMP receptor 1A prevents the harmful effect of exogenous BMP4 (Wong *et al.* 2010c), demonstrating their importance in the response. The role of BMP4 in stimulating COX-2 expression by increasing ROS production has also been confirmed in arteries of hypertensive rats and humans since noggin, a BMP4 antagonist, improves endothelial function and reduces BMP4 and COX-2 expression in renal arteries of hypertensive rats and patients (Wong *et al.* 2010c).

Obesity and PVAT—Adipose tissues can produce ROS and thus contribute to endothelial dysfunction (Gao *et al.* 2006, Wang *et al.* 2014a; see section Nitric oxide). NOX subunits are localized in the cytoplasm and cell membrane of the adipocytes of PVAT (Gao *et al.* 2006, Wang *et al.* 2014a). Superoxide anion production by PVAT is inhibited by diphenylene iodonium (DPI) and enhanced by the stimulator of the enzyme, NADH (Gao *et al.* 2006). Stimulated superoxide anion formation in PVAT may reduce the bioavailability of endothelium-derived NO, thereby mediating an increase in vascular tone (Wang *et al.* 2014a). PVAT-derived ROS also modulate the release of adipokines and enhance the responsiveness of vascular muscle cells to EDCF by reducing the recycling of TP receptors (Valentin *et al.* 2003, Wang *et al.* 2004). Finally, they impair adiponectin signalling that normally enhances NO generation and endothelium-dependent relaxations (Wang *et al.* 2014a; see section Nitric oxide). Thus, ROS produced by PVAT enhance endothelium-dependent contractions. Besides producing ROS, adipose tissues, in particular PVAT, can facilitate contractions of the vascular smooth muscle cells that they surround by releasing adipocyte-derived contracting mediators, including lipocalin-2, resistin, calpastatin, chemerin, angiotensinogen, vasoconstrictor prostanoids, superoxide anions and ET-1 (Gollasch 2012, Gu & Xu 2013, Meyer *et al.* 2013, Oriowo 2015). Of those, *lipocalin-2* is an important contributor to endothelial dysfunction and the emergence of endothelium-dependent contractions, in particular. Lipocalin-2 production is upregulated in obese human subjects and animals (Wang 2012). Augmented circulating lipocalin-2 levels have also been found in patients with cardiovascular abnormalities (Hemdahl *et al.* 2006). Polyamination of lipocalin-2 facilitates its clearance from the circulation (Song *et al.* 2014). However, lipocalin-2 can undergo deamidation, presumably within adipose tissues (Song *et al.* 2014). Once it is deaminated, the circulating half-life of the deleterious adipokine is enhanced and the high levels of the deaminated form exert pro-inflammatory and endothelial damaging effects (Song *et al.* 2014). Mice deficient in lipocalin-2 are protected against endothelial dysfunction caused by dietary challenges (Liu *et al.* 2012a). Compared with those of wild type controls, arteries of lipocalin-2 knockout mice exhibit a longer NO bioavailability and increased responsiveness to endothelium-dependent vasodilators (Liu *et al.* 2012a). Deficiency of this adipokine results in diminished EDCF-mediated contractions (Liu *et al.* 2012a). Administration of exogenous lipocalin-2 to lipocalin-2 knockout mice attenuates endothelium-dependent relaxations to insulin and promoted endothelium-dependent contractions to acetylcholine by promoting eNOS uncoupling and COX overexpression respectively (Liu *et al.* 2012a).

High-fat intake and obesity potentiate the occurrence of EDCF-mediated responses, possibly because of insulin resistance, resulting in greater production of oxygen-derived free radicals, an upregulation of the expression of TP receptors, and the unleashed production of ET-1 (see section Hypoxia: when NO turns bad) (Traupe *et al.* 2002a,b, Mundy *et al.* 2007, Xiang *et al.* 2008, Gollasch 2012). The potentiation of endothelium-dependent contractions in obesity, besides the obvious role of PVAT, can be attributed to

TLR4 activation by saturated fatty acids and/or bacterial endotoxin lipopolysaccharides (Liang *et al.* 2013). Indeed, deletion of TLR4 protects mice against endothelial dysfunction resulting from obesity, whether genetically or diet-induced both by potentiating endothelium-dependent relaxations (see section Nitric oxide) and decreasing endothelium-dependent contractions (Liang *et al.* 2013). Activation of TLR4 promotes the transcription of NOX-1 and NOX-4, resulting in elevated production of ROS; the increased level of ROS favours eNOS uncoupling, decreases NO production and bioavailability, impairs EDH-mediated responses and increases the activity of COX1 with augmented EDCF-mediated contractions (Liang *et al.* 2013).

Hallmark of disease. Diabetes—The endothelium-dependent relaxations to acetylcholine are blunted in a number of arteries from diabetic animals (Tesfamariam 1994, De Vriese *et al.* 2000). This is due in part to the concomitant release of EDCF and can be attributed to the exposure of the endothelial cells to high glucose, resulting in increased oxidative stress and overexpression not only of both COX-1 and COX-2 but also of TP receptors in vascular smooth muscle (Tesfamariam *et al.* 1990, 1991, Shi *et al.* 2006, 2007a,b, 2008, Xu *et al.* 2006, Obrosova *et al.* 2007, Michel *et al.* 2008, Shi & Vanhoutte 2008, Ramos-Alves *et al.* 2012, Zhu *et al.* 2014). In the case of diabetes, the production of ROS plays a crucial role in triggering and amplifying EDCF-mediated responses (Shi *et al.* 2007b, 2008, Shi & Vanhoutte 2008).

Hypertension—The endothelium-dependent relaxations to acetylcholine are blunted and the endothelium-dependent contractions to acetylcholine are more pronounced in arteries of the SHR than in those of normotensive WKY (Lockette *et al.* 1986, Lüscher & Vanhoutte 1986, Lüscher *et al.* 1987b,c, Koga *et al.* 1989, Kähönen *et al.* 1998). These changes are prevented by inhibitors of COX and antagonists at TP receptors (Lüscher & Vanhoutte 1986, Koga *et al.* 1989, Kung & Lüscher 1995, Zhou *et al.* 1999, Yang *et al.* 2003). Increased production of vasoconstrictor prostaglandins also contributes to the endothelial dysfunction evoked by increased circulating levels of aldosterone (Xavier *et al.* 2008). The increase in intracellular endothelial calcium concentration caused by acetylcholine is greater in SHR arteries than in those of the WKY, while during exposure to A23187 it is comparable, suggesting that a key aspect of the prominence of endothelium-dependent contractions in the former relates to an abnormal handling of calcium (Tang *et al.* 2007). In addition, in the aorta of the hypertensive strain the expression of COX-1 is increased (Ge *et al.* 1995, Tang & Vanhoutte 2008b). However, this overexpression is not present in arteries of pre-hypertensive SHR (Ge *et al.* 1999, Tang & Vanhoutte 2008b). These findings prompt the conclusion that the overexpression of the enzyme in arteries from adult hypertensive animals reflects premature ageing of the endothelium rather than a genetic predisposition. The burst of endothelial free radicals is also greater in arteries of the SHR than in those of the WKY (Tang *et al.* 2007), implying a greater facilitation of EDCF-mediated responses. The expression of the prostacyclin synthase gene is more abundant in endothelial cells of the SHR than in the WKY endothelium, and the protein presence of the enzyme is augmented by hypertension (Numaguchi *et al.* 1999, Tang & Vanhoutte 2008b). These endothelial changes explain why acetylcholine causes a greater release of endoperoxides and prostacyclin in SHR than in WKY arteries (Ge *et al.* 1995, Gluais *et al.* 2005). In addition, although the mRNA expression and protein presence of TP receptors are comparable in arteries of WKY and SHR (Tang & Vanhoutte 2008b, Tang *et al.* 2008), the latter are hyper-responsive to the vasoconstrictor effect of endoperoxides and prostacyclin (Levy 1980, Ge *et al.* 1995, Rapoport & Williams 1996, Gluais *et al.* 2005). This hyper-responsiveness is present in pre-hypertensive animals, and thus, the hyper-responsiveness is not a consequence of premature ageing caused by the chronic exposure to increased blood pressure (Ge *et al.* 1999). However, certain genetic traits (in particular the absence of IP receptor responsiveness) may further aggravate endothelial dysfunction. Obviously, the absence of vasodilator response to prostacyclin contributes, and helps to explain why in humans cardiovascular disease is accelerated by a dysfunctional prostacyclin receptor mutation (Arehart *et al.* 2008) (Fig. 11). Aspirin and indomethacin potentiate the vasodilator response to acetylcholine in the forearm of patients with hypertension but not in that of normotensive subjects (Taddei *et al.* 1995b, 1997a,b, Monobe *et al.* 2001). This then suggests that EDCF-mediated responses also are part of the endothelial dysfunction of human hypertension.

Coronary disease—Aspirin and the TP receptor inhibitor terutroban improve responses to endothelium-dependent vasodilator stimuli in patients with CAD, suggesting that endothelium-derived prostanoids contribute to the endothelial dysfunction resulting from the disease (Husain *et al.* 1998, Belhassen *et al.* 2003).

Immunodeficiency—Subjects with human immunodeficiency virus (HIV) infection exhibit vascular inflammation and endothelial dysfunction to judge from reduced

flow-mediated vasodilatations (Blanco *et al.* 2006). These complications are related both to the HIV infection and the highly active antiretroviral therapy that patients are placed on (Friis-Møller *et al.* 2007, Hulten *et al.* 2009). The endothelial dysfunction observed in HIV-infected patients is explained best by an increase microvascular production of ROS, thereby reducing NO bioavailability, blunting acetylcholine-induced endothelium-dependent relaxation, facilitating endothelium-dependent contractions and enhancing TP receptor responsiveness (Wang *et al.* 2013).

Endothelin-1

Production, actions and clearance of endothelin-1. ET-1, the first member of the endothelin peptide family originally identified in endothelial cells (Yanagisawa *et al.* 1988), is a potent vasoconstrictor peptide and the most abundant isoform in the cardiovascular system (Kurihara *et al.* 1994, Schiffrin 1999). ET-1 is synthesized from a precursor, the pre-proET-1 which is successively cleaved into the basically inactive 39 amino acid Big-ET-1. Then, Big-ET-1 is converted to the 21-amino acid ET-1, predominantly but not exclusively, by the action of endothelin-converting enzymes (ECEs), belonging to the neprilysin family (D'Orléans-Juste *et al.* 2003, Kohan *et al.* 2011).

ET-1 interacts with two G protein-coupled receptors termed ET_A and ET_B (Arai *et al.* 1990, Sakurai *et al.* 1990, Masaki *et al.* 1994). Both ET_A and ET_B receptors are localized on vascular smooth muscle cells where they exert their vasoconstrictor, proliferative and hypertrophic action, but in arteries, the former are the predominant vasoconstrictor receptor (Rubanyi & Polokoff 1994). In the vascular wall, ET_B receptors are expressed predominantly in the endothelial cells and its activation is associated with the release of the anti-aggregator, vasodilator and antiproliferative NO and prostacyclin (De Nucci *et al.* 1988, Thorin & Clozel 2010). Both *in vivo* and *in vitro*, these endothelial effects of ET-1 counterbalance the effects of ET_A/ET_B stimulation on the vascular smooth muscle cells (Fig. 12). Besides the endothelial cells, the ET_B receptor is highly expressed in the renal collecting duct where it contributes to the regulation of sodium excretion and therefore of arterial blood pressure (Kohan *et al.* 2011).

Endothelins are degraded locally, at least in part by neutral endopeptidase (NEP) and deamidase, and are rapidly eliminated from the circulation by binding to the endothelin ET_B receptor subtype, here acting as a clearance receptor (Thorin & Clozel 2010, Kohan *et al.* 2011). The rapid clearance of ET-1 by the pulmonary, splanchnic and renal circulations, associated with its local processing, implies that ET-1 is an autocrine or paracrine mediator rather than a systemic hormone (Culshaw *et al.* 2015).

Regulation of production and action of endothelin-1. Under normal physiological conditions, the endothelial production of ET-1 and/or its action on vascular smooth muscle is tightly kept under control by counter-regulatory systems. Indeed, contractions and pressor response initiated by ET-1 are unusual when compared to those produced by most other vasoconstrictors in that they are slowly developing and long lasting even after washing out the peptide (De Nucci *et al.* 1988, Yanagisawa *et al.* 1988).

NO, the gatekeeper—Stimulation of NO production inhibits the expression and the production of endothelin-1 (Boulanger & Lüscher 1990, Vanhoutte 2000). After translation and processing in the endothelial cells, ET-1 is usually secreted immediately. Intracellular storage and on-demand release can occur, but it is rather uncommon (Goel *et al.* 2010). Whether or not NO acts on the former or the latter releasing pathway is basically unknown. Furthermore, the powerful and sustained vasoconstriction elicited by this peptide is efficiently blunted by both exogenous and endothelium-derived NO, in a cyclic GMP-dependent manner (Miller *et al.* 1989, Lillestol *et al.* 1998). Finally, when released, endothelin-1 activates nearby endothelial ET_B receptors, which are associated to NO production, including in human coronary arteries (Schini *et al.* 1991, Halcox *et al.* 2007). Thus, under normal conditions, any overproduction of ET-1 would be offset by the increased release of NO, which downregulates the generation of the peptide and curtails its vasoconstrictor and growth-stimulating effects (Vanhoutte 2000, Vanhoutte *et al.* 2009, De Mey & Vanhoutte 2014; Fig. 12). *In vivo*, the sustained increase in arterial blood pressure caused by the acute or chronic administration of various NOS inhibitors is reduced by ET_A antagonists, further substantiating the preponderant role of NO in regulating the release and the vasoconstrictor action of ET-1 (Banting *et al.* 1996, Gardiner *et al.* 1996, Marjan *et al.* 1998, Pollock 1999).

Calcitonin gene-related peptide—Calcitonin gene-related peptide is expressed mainly in neurons and endocrine cells (Brain & Grant 2004). Contractile responses to ET-1 are sensitive to the relaxing effects of CGRP, whether administrated exogenously or released from perivascular sensory nerves by capsaicin, a vanilloid molecule, acting on TRPV1 (Félétou and Vanhoutte 2006c, Meens *et al.* 2009, 2010). GCRP also stimulates NO-mediated endothelium-dependent

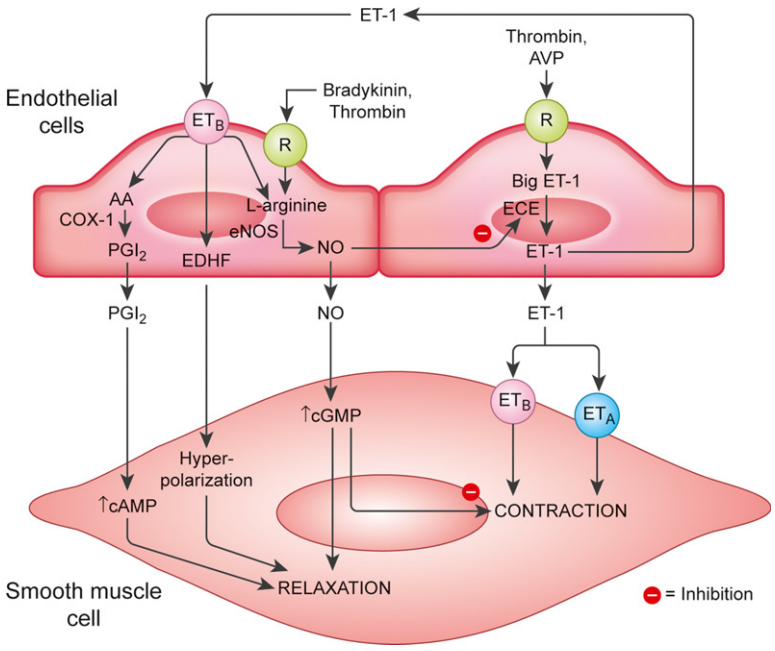

Figure 12 NO and endothelin. Release and actions of endothelin-1 (ET-1) in the vascular wall. AA, arachidonic acid; AVP, arginine vasopressin; cAMP, cyclic AMP; cGMP, cyclic GMP; COX, cyclooxygenases; ECE, endothelin converting enzyme; ET_A and ET_B, endothelin receptors; NO, nitric oxide; NOS, nitric oxide synthase; PGI_2, prostacyclin; R, cell membrane receptor.

vasodilatation as well as cAMP production by adenylyl cyclase in the vascular smooth muscle cells (Brain & Grant 2004). However, termination of the long-lasting interaction between ET-1 and ET_A receptor (and thus of the sustained contraction induced by the peptide) does not involve these pathways, but rather CGRP receptor-dependent G protein $\beta\gamma$ subunit-mediated signalling (Meens *et al.* 2012). In line with the varying distribution of sensorimotor nerves, in blood vessels this CGRP/ET_A receptors crosstalk is not uniformly distributed in all vascular smooth muscle (Meens *et al.* 2011), but is present in human coronary arteries (Labruijere *et al.* 2013) and in rats *in vivo* (Meens *et al.* 2011). Therefore, besides NO, the feedback inhibition of the action of ET-1 by CGRP is sufficiently important to affect peripheral vascular resistance and arterial blood pressure in responses to ET-1 (Meens *et al.* 2011). The physiological relevance of a potential regulation of the action of ET-1 by sensorimotor nerves, the most likely vascular source of CGRP, remains to be assessed (De Mey & Vanhoutte 2014). Nevertheless, TRPV1 activation exerts a protective effect in hypertension and its associated disorders indicating that it counteracts vascular dysfunction (Zhang *et al.* 2015).

Physiological role of ET-1 in the peripheral and coronary circulation. Although ET-1 has been described as one of the most potent known vasoconstrictors, its acute administration in healthy mammals, including humans, leads to a biphasic response, an initial and transient reduction in arterial blood pressure followed by a sustained hypertensive phase, due to the primary activation of endothelial ET_B receptors followed by that of vasoconstrictor (primarily ET_A and to a lesser extent ET_B) receptors on the vascular smooth muscle (Kurihara *et al.* 1994, Haynes *et al.* 1995a,b, Thorin & Webb 2010).

Lessons from genetically modified animals—Knocking out preproET-1, endothelin-converting enzyme 1 or ET_A receptor leads to lethality in homozygous animals and therefore does not allow a proper appraisal of the role of ET-1 in cardiovascular physiology (Von Websky *et al.* 2009). The arterial blood pressure of heterozygous knockout mice for the ET_A receptor is in the normal range, while heterozygous ET_B knockout mice are hypertensive (Berthiaume *et al.* 2000). Specific deletion of the ET_B receptor in endothelial cells or in the renal collecting duct shows that the hypertension can be attributed to an enhanced sodium retention but not to an increased vascular resistance, an interpretation substantiated by the hypertensive phenotype also observed in mice with specific deletion of ET-1 in the collecting duct (Kohan 1996, Ahn *et al.* 2004, Ge *et al.* 2005, 2006, Bagnall *et al.* 2006). Transgenic mice overexpressing ET-1 are normotensive but exhibit renal fibrosis leading to fatal kidney disease (Hocher *et al.* 1997). In such mice hypertension occurs only after salt loading (Hocher *et al.*

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1997); they show exacerbated vasoconstriction and exaggerated increases in arterial blood pressure with concomitant NOS inhibition or deletion (Von Websky *et al.* 2009). Mice with specific conditional deletion of the ET-1 gene in their endothelial cells are hypotensive (Kisanuki *et al.* 2010). Conversely, transgenic mice strongly overexpressing the human ET-1 gene selectively in the endothelium exhibit an ET_A-dependent increase in arterial blood pressure (Leung *et al.* 2011, Rautureau *et al.* 2015), while less severe ET-1 overexpression causes only vascular injury, attributable to early changes in the expression of genes associated with enhanced lipid biosynthesis, but no elevation in arterial blood pressure (Simeone *et al.* 2011).

Thus, these studies on genetic murine models emphasize the importance of ET-1 and ET_B receptors in controlling sodium and fluid homeostasis in the kidney while in the vasculature they provide evidence for the existence of a paracrine vasoregulator pathway mediated by endothelial-derived ET-1 acting on the vascular smooth muscle ET_A receptors and illustrate the predominant role of the NO system in regulating ET-1 synthesis and action.

Lessons from pharmacological experiments—The contribution of endogenous ET-1 generation to human cardiovascular homeostasis has generally been assessed by acute or short-lasting administration of antagonists of ET_A and/or ET_B receptors. In healthy humans, systemic inhibition of ET_B increases peripheral resistance and arterial blood pressure (Strachan *et al.* 1999). In most of the studies involving healthy subjects, the peripheral administration of an ET_A antagonist (generally BQ123) increases forearm blood flow (Love *et al.* 1996, McAuley *et al.* 2000, Boak *et al.* 2005), a response blunted by either an ET_B antagonist or a NOS inhibitor (Verhaar *et al.* 1998). However, such vasodilator response to ET_A blockade is not observed consistently (Cardillo *et al.* 2002a,b, 2004, Campia *et al.* 2004, Westby *et al.* 2011). Similarly, phosphoramidon, a mixed ECE/NEP inhibitor, but not thiorphan a selective NEP inhibitor, increases forearm blood flow (Haynes & Webb 1994, Haynes *et al.* 1995b, Love *et al.* 1996). These results suggest that the constitutive production and release of ET-1, via a balanced action on both its receptors, contribute to the regulation of regional blood flow and arterial blood pressure. The observation that *in vivo* ET_B receptor activation exerts a tonic effect is rather surprising since vasodilatation associated to the release of endothelium-derived NO in response to exogenous and acute administration of ET_B agonists are highly transient (Newby *et al.* 2002) and since stimulated ET_B receptors internalize rapidly (Wu-Wong *et al.* 1995). Nevertheless, under physiological conditions, blockade of ET-1 receptors overall has little or moderate effects, by contrast to what is observed in pathological situations (Iglarz & Clozel 2010).

Compared to peripheral arteries the coronary circulation appears more prone to the vasoconstrictor effects of ET-1 (Kolettis *et al.* 2013). In subjects with angiographically normal coronary arteries, BQ123 (ET_A receptor antagonist) induces an increase in the diameter of the proximal segments (Kyriakides *et al.* 2000, Kinlay *et al.* 2001, MacCarthy *et al.* 2001), and in coronary patients, mixed ET_A/ET_B blockade also increases the diameter of coronary vessels with no or mild angiographic alterations (Wenzel *et al.* 1998, Halcox *et al.* 2007). However, selective ET_B receptor antagonism causes coronary microvascular constriction, without affecting tone of the epicardial arteries, by reducing endothelin clearance and NO bioavailability (Halcox *et al.* 2007).

Hallmark of disease. Hypoxia—Hypoxia is a major positive regulator of ET-1 synthesis, increasing the gene expression of the peptide in animal in animal and human endothelial cells and augmenting its release from various vascular beds *in vivo* and thus its circulating levels (Rakugi *et al.* 1990, Kourembanas *et al.* 1991, Elton *et al.* 1992).

Diabetes—Hyperglycaemia enhances the expression of ET-1 and its constitutive production by endothelial cells (Yamauchi *et al.* 1990, Park *et al.* 2000) and plasma ET-1 levels are elevated in patients with diabetes (Schneider *et al.* 2002). In peripheral and coronary arteries of various diabetic animals (Verma *et al.* 2002, Ergul 2011), as well as in isolated peripheral arteries of patients with diabetes (McIntyre *et al.* 2001), hyper-responsiveness to ET-1 is obvious. Furthermore, in patients with diabetes, endogenous ET-1 contributes to the maintenance of basal vascular tone and to endothelial dysfunction (Cardillo *et al.* 2002a, Mather *et al.* 2002, Yoon *et al.* 2008, Rafnsson *et al.* 2012). In these patients, selective ET_A and dual ET_A-ET_B antagonists improve endothelium-dependent vasodilatations, while ET_B blockade alone increases basal blood flow without improving endothelium-dependent responses (Rafnsson *et al.* 2014).

Hypertension—ET-1 may be involved in the pathogenesis of hypertension, especially in salt-dependent hypertension (Moorhouse *et al.* 2013). Indeed, ET-1 is a potent vasoconstrictor in the kidney and thus can have opposing actions on water and sodium regulation. If ET_B stimulation in the collecting duct prevents water and sodium reabsorption and therefore volume expansion, ET-1 contributes also to the tone of the glomerular afferent and efferent arterioles. A prolonged

vasoconstriction of these arteries, predominantly via ET_A activation, produces a decrease in renal blood flow and a reduction in glomerular filtration rate associated with an enhanced filtration fraction, leading to sodium and water reabsorption (Laffin & Bakris 2015). Additionally, ET-1 increases vascular ROS formation, is a pro-inflammatory and pro-fibrotic agent and promotes vascular remodelling. These phenomena are strongly associated with hypertension and endothelial dysfunction (Sandoval *et al.* 2014).

In deoxycorticosterone acetate (DOCA)-salt hypertensive animals, the levels of both ET-1 and vascular superoxide anions are elevated and ET_A blockade decreases arterial blood pressure and normalizes ROS production (Callera *et al.* 2003). However, the source of reactive species generation either by xanthine oxidase, mitochondrial enzymes and/or NOX are uncertain (Beswick *et al.* 2001, Li *et al.* 2003, Viel *et al.* 2008).

Patients with hemangioendothelioma have increased serum levels of ET-1 and elevated arterial blood pressure; both normalized following tumour removal (Yokokawa *et al.* 1991). However, in hypertensive patients, plasma levels of ET-1 are not necessarily different from those in healthy subjects, possibly because of the vectorization of ET-1 release towards the abluminal side of the endothelial cells and the efficiency of the clearance system (Goddard & Webb 2000). Additionally, the endothelial damage caused by a rise in arterial blood pressure augments ET-1 expression in blood vessels and in the heart. The peptide can also be upregulated by a number of factors involved in vascular disease (Iglarz & Clozel 2007). Thus, the involvement of the endothelin system could be secondary to the chronic increase in arterial blood pressure, rather than a primary in essential hypertension (Schiffrin 2005).

Nevertheless, endothelin antagonists increase forearm blood flow and reduce arterial blood pressure to a greater extent in hypertensive than in normotensive subjects (Goddard & Webb 2000). Both mixed ET_A/ET_B (Krum *et al.* 1998) and more selective ET_A (Moorhouse *et al.* 2013) antagonists are efficacious in doing so. However, these agents are not used for the treatment of primary hypertension since their side effects, predominantly the occurrence of peripheral oedema, outweigh their therapeutic benefit (Laffin & Bakris 2015).

Endothelin antagonists are prescribed for the treatment of pulmonary hypertension (Steriade *et al.* 2014) and could be of interest in resistant hypertension, in hypertension associated with chronic kidney disease or metabolic syndrome (Moorhouse *et al.* 2013, Laffin & Bakris 2015), or in patients with drug-dependent increases in arterial blood pressure [e.g. angiogenesis inhibitor (Lankhorst *et al.* 2014) or calcineurin inhibitor (Raina *et al.* 2012)]. Additionally, animal studies indicate that ET-1 is likely to play a major contributing role in the genesis of preeclampsia (Palei *et al.* 2013). However, the teratogenic effects of ET-1 deletion (Kurihara *et al.* 1994) make this therapeutic indication for endothelin antagonist unlikely, unless proven safe when administrated at the end of pregnancy.

Atherosclerosis and coronary disease—ET-1, beyond its function as a vasoactive peptide, also plays a crucial role in the atherogenic process by enhancing mitogenesis, inducing extracellular matrix formation and contributing to the development of inflammation within the blood vessel wall (Schiffrin 1999). Hence, ET-1 has been implicated in the generation of atherosclerosis (Bacon *et al.* 1996) and the pathophysiology of numerous coronary artery disorders, including coronary endothelial dysfunction (Lerman *et al.* 1995), coronary spasm (Toyo-oka *et al.* 1991), myocardial infarction (Miyauchi *et al.* 1989) and myocardial reperfusion injury (Tamareille *et al.* 2013). ET-1 induces endothelial dysfunction by interference with glucose and lipid metabolism, increased oxidative stress, disrupting the NO pathway, and accelerating inflammatory processes (Kolettis *et al.* 2013).

There is controversy regarding peripheral vascular responses to endogenous or exogenous ET-1 in hypercholesterolaemic patients, being either unaltered (Nohria *et al.* 2003, Boak *et al.* 2005) or enhanced (Cardillo *et al.* 2000). However, in patients with atherosclerosis, the contribution of endogenous ET-1 to the active constriction of coronary arteries is augmented, especially at the sites of stenosis (Kinlay *et al.* 2001). Long-term endothelin receptor antagonism improves endothelial function and attenuates plaque progression in patients with early atherosclerosis (Reriani *et al.* 2010, Yoon *et al.* 2013).

Myocardial infarction—Plasma levels of ET-1 are increased in various animal models of acute myocardial infarction (Kolettis *et al.* 2013). In clinical studies, they correlate with infarct size (Miyauchi *et al.* 1989, Yasuda *et al.* 1990) and predict short or long-term outcome (Omland *et al.* 1994, Yip *et al.* 2005). Additionally, ET-1 is released during the early phase of reperfusion (Malatino *et al.* 1993) and contributes to myocardial reperfusion injury by activating ET_A receptors (Tamareille *et al.* 2013). In patients with ST segment elevation and acute myocardial infarction, ET_A blockade prior to reperfusion improves myocardial perfusion, decreases infarct size and improves long-term outcome (Adlbrecht *et al.* 2012, 2014).

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Heart failure—ET-1 can drive the progression of heart failure. It is a potent constrictor of both systemic and pulmonary arterioles and veins and is involved in vascular and myocardial hypertrophy and fibrosis. Thus, in patients with heart failure, ET-1 contributes importantly to peripheral resistance, and the activation of both ET_A and ET_B receptors causes vasoconstriction (Love *et al.* 1996). In these patients, circulating concentrations of precursor BigET-1, or those of the active peptide itself are enhanced and constitute independent predictors of morbidity and mortality (Wei *et al.* 1994, Masson *et al.* 2006, Gottlieb *et al.* 2015). Short-term ET-1 receptor blockade in patients with severe heart failure have hemodynamic benefits (decrease in systemic and pulmonary resistance as well as in atrial pressure and an increase in cardiac output). However, the results of chronic clinical trials with either mixed ET_A-ET_B or specific ET_A receptor antagonists in both acute and chronic heart failure were disappointing (Kohan *et al.* 2012).

Coronary restenosis—ET-1, acting on ET_A receptors, promotes neo-intimal lesion formation and luminal occlusion following vascular injury. The expression of ET-1, ECE and endothelin receptors are enhanced in neo-intimal vascular smooth muscle cells after percutaneous coronary angioplasty (Shirai *et al.* 2006). Additionally, vascular remodelling in human internal mammary arteries (used for coronary bypass grafting) is associated with enhanced presence of ET-1 and augmented expression of ET_A and to a lesser extent of ET_B receptors (Sutherland *et al.* 2006). The beneficial effects of ET receptor blockade can already be observed even before grafting since ET_A, ET_B and mixed ET_A-ET_B antagonists improve endothelium-dependent relaxations in human internal mammary arteries (Verma *et al.* 2001). Again, at least in rodents, ET_A receptors are predominantly involved in this deleterious effect of ET-1, since selective ET_A antagonism reduces neo-intimal lesion size in a mouse model of intraluminal injury (Duthie *et al.* 2015).

Hypoxia: when NO turns bad

In a number of isolated arteries and veins, hypoxia causes an acute contraction of vascular smooth muscle that is more pronounced when the preparations are constricted and is therefore termed *hypoxic augmentation* of vasoconstriction. Most of the hypoxic augmentation is endothelium dependent (De Mey & Vanhoutte 1982, 1983) and involves the transfer of a chemical mediator (Rubanyi & Vanhoutte 1985, Iqbal & Vanhoutte 1988). The endothelium-dependent component of the response requires the presence of NO and activation of sGC; it does not require increased levels of cGMP, the prototypical product of sGC (Gräser & Vanhoutte 1991, Pearson *et al.* 1996, Chan *et al.* 2011, Chen *et al.* 2014). However, this hypoxic endothelium-dependent response is accompanied by increases in the intracellular level of inosine 5′-triphosphate (ITP) and in the synthesis of inosine 3′,5′-cyclic monophosphate (cIMP) by sGC (Beste *et al.* 2012, Chen *et al.* 2014). The administration of either exogenous cIMP or ITP causes augmented vasoconstriction to hypoxia (Chen *et al.* 2014). The contractions evoked by hypoxia and cIMP are associated with increased activity of Rho-kinase, implying that cIMP mediates the hypoxic effect by sensitizing the myofilaments of the vascular smooth muscle cells to calcium via activation of Rho-kinase (Chan *et al.* 2011, Chen *et al.* 2014). Hypoxic vasoconstriction is exacerbated in the coronary circulation by previous ischaemia–reperfusion injury (Pearson *et al.* 1996). Since hypoxia is implicated in the exaggerated coronary vasoconstrictions accompanying CAD, myocardial infarction, hypertension and stroke, the newly found role of cIMP may help to identify unique therapeutic targets for certain cardiovascular disorders, in particular those associated with sleep apnoea (Gao & Vanhoutte 2014, Gao *et al.* 2014, Leung *et al.* 2015).

Conclusion

Native, healthy endothelial cells respond to a number of stimuli (e.g. serotonin from aggregating platelets and thrombin) by releasing NO, which relaxes the vascular smooth muscle that surrounds them (except under hypoxic conditions when it favours vasoconstriction). NO, in synergy with prostacyclin, further inhibits platelet aggregation. It also reduces the endothelial expression of adhesion molecules and thus the adhesion and penetration of leucocytes (macrophages). The endothelial mediator also prevents the proliferation of vascular smooth muscle cells and limits the formation of oxyLDL. Ageing and certain lifestyle factors (e.g. lack of exercise, Western diet, pollution and smoking), or certain diseases (e.g. diabetes and hypertension) result in a lesser release of NO and an acceleration of the turnover of the apoptotic process in the endothelium. The apoptotic endothelial cells are replaced by regenerated ones. However, such regenerated cells are dysfunctional, senescent, and incapable of producing the required amounts of NO, which facilitates the inflammatory response leading to the formation of atherosclerotic plaques. The shortage of NO also unleashes the production not only of endothelium-derived vasoconstrictor prostanoids (in particular endoperoxides, and prostacyclin) but also that of ET-1. These mediators activate receptors of the vascular smooth muscle cells

leading to vasoconstriction which amplifies the degree of endothelial dysfunction. The understanding of the delicate balance between vasodilator (growth inhibiting) and vasoconstrictor (growth stimulating) signals emitted by endothelial cells is far from complete. In particular, how this balance varies in blood vessels of different sizes in diseased subjects is pretty much an open question. Indeed, we know a lot about endothelial dysfunction in large arteries (the obvious focus of this review) but only begin to appreciate the role of the endothelium in pathologies involving the smaller arteries and the microcirculation.

Conflict of interest

There is no conflict of interest to declare.

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