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REVIEW

Endothelial dysfunction and vascular disease

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Abstract

The endothelium can evoke relaxations (dilatations) of the underlying vascular smooth muscle, by releasing vasodilator substances. The best characterized endothelium-derived relaxing factor (EDRF) is nitric oxide (NO). The endothelial cells also evoke hyperpolarization of the cell membrane of vascular smooth muscle (endothelium-dependent hyperpolarizations, EDHF-mediated responses). Endothelium-dependent relaxations involve both pertussis toxin-sensitive G_i (e.g. responses to serotonin and thrombin) and pertussis toxin-insensitive G_q (e.g. adenosine diphosphate and bradykinin) coupling proteins. The release of NO by the endothelial cell can be up-regulated (e.g. by oestrogens, exercise and dietary factors) and down-regulated (e.g. oxidative stress, smoking and oxidized low-density lipoproteins). It is reduced in the course of vascular disease (e.g. diabetes and hypertension). Arteries covered with regenerated endothelium (e.g. following angioplasty) selectively loose 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 causing endothelium-dependent hyperpolarizations), endothelial cells also can evoke contraction (constriction) of the underlying vascular smooth muscle cells by releasing endothelium-derived contracting factor (EDCF). 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. EDCF-mediated responses are exacerbated when the production of NO is impaired (e.g. by oxidative stress, ageing, spontaneous hypertension and diabetes). They contribute to the blunting of endothelium-dependent vasodilatations in aged subjects and essential hypertensive patients.

Keywords cyclooxygenase, diabetes, G-proteins, hypertension, nitric oxide, prostanoids.

The seminal observation of Robert Furchgott demonstrated that the removal of the endothelial layer from isolated arteries prevents the *in vitro* dilator response to acetylcholine (Furchgott & Zawadzki 1980). This simple experiment has profoundly modified our thinking about the local control of vasomotor tone. Early bioassay studies demonstrated that the endothelial cells cause arterial relaxation by releasing a powerful vasoactive substance(s) which was termed endothelium-derived relaxing factor (EDRF) (Fig. 1) (Furchgott &

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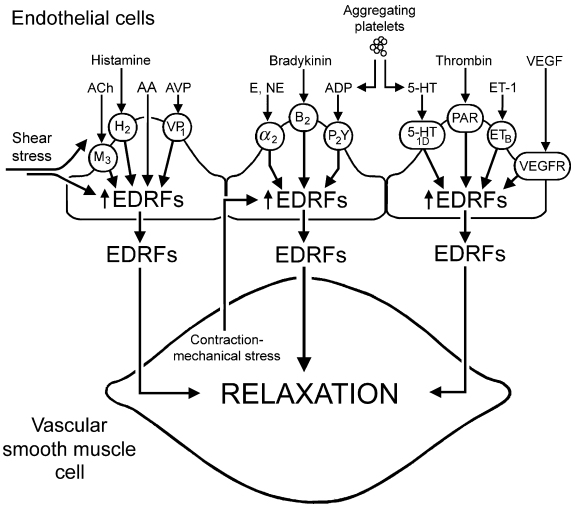

Figure 1 Some of the neurohumoral mediators that cause the release of endothelium-derived relaxing factors (EDRF) through activation of specific endothelial receptors (circles). A, adrenaline (epinephrine); AA, arachidonic acid; Ach, acetylcholine; ADP, adenosine diphosphate; α, alpha adrenergic receptor; 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; P, purinergic receptor; T, thrombin receptor; VEGF, vascular endothelial growth factor; VP, vasopressin receptor.

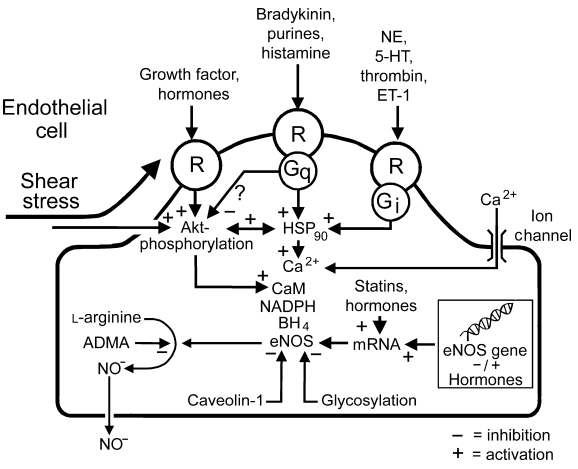

Figure 2 Schematic of possible mechanisms by which production of nitric oxide is regulated in endothelial cells. Nitric oxide is produced through enzymatic conversion of L-arginine by nitric oxide synthase (endothelial or type III, eNOS). The transcription of this enzyme is regulated genomically by hormones and growth factors. Stability of eNOS mRNA is modulated by statins and hormones. eNOS enzyme activity requires calcium, calmodulin, nicotinamide adenine dinucleotide phosphate (NADPH) and 5,6,7,8-tetra-hydrobiopterine (BH_4). Enzyme activity is regulated by complexing to 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 to pertussis toxin (G_i) or insensitive to pertussis toxin (G_q), activate intracellular pathways that modulate eNOS activity post-translationally through heat shock protein 90 or AKT phosphorylation. Association of eNOS with caveolin-1 or glycosylation of the enzyme reduces activity. A metabolite of L-arginine, asymmetric dimethyl arginine (ADMA), decreases production of the nitric oxide through competitive binding to eNOS. Thus, this endogenous amine may be a risk factor for the development of cardiovascular disease. +, stimulation; –, inhibition; ?, pathways in which the regulation is unknown (modified from O'Rourke *et al.* 2006).

Zawadzki 1980, Rubanyi *et al.* 1985). The original EDRF (Furchgott & Zawadzki 1980) stimulates soluble guanylyl cyclase in the vascular smooth muscle cells and thus increases the production of cyclic guanosine monophosphate (see Ignarro *et al.* 1986, Furchgott & Vanhoutte 1989, Lüscher & Vanhoutte 1990). It is rapidly destroyed by superoxide anions (Gryglewski *et al.* 1986, Rubanyi *et al.* 1986). These experimental facts led to the proposal (Furchgott 1988, Ignarro *et al.* 1988a,b) and the demonstration (Palmer *et al.* 1987, 1988a,b, Palmer & Moncada 1989, Moncada 1997) that EDRF is nitric oxide (NO) (Fig. 2). However, the release of NO is by no means the only way to evoke endothelium-dependent vasomotor changes. Thus, besides NO, a number of endothelium-derived factors (EDHFs) and the opening of gap junctions can cause NO-independent hyperpolarizations of the underlying vascular smooth muscle (Fig. 3) (see Busse *et al.* 2002, Félétou & Vanhoutte 2006a,b, 2007, Fleming & Busse 2006). In addition, endothelial cells can release vasoconstrictor prostanoids (endothelium-derived contracting factors, EDCF) (Fig. 4) (see Furchgott & Vanhoutte 1989, Lüscher & Vanhoutte 1990, Vanhoutte 1993a, Vanhoutte *et al.* 2005). When the ability of the endothelial cells to release NO (and to cause endothelium-dependent hyperpolarizations) 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 (see Vanhoutte 1988, 1996, 1997, 2002, 2003, Vanhoutte & Shimokawa 1989, Shepherd & Vanhoutte 1991, Félétou & Vanhoutte 2006c). Thus, endothelial dysfunction has become a hallmark, and indeed a predictor of cardiovascular disease (e.g. Lyons 1997, Behrendt & Ganz 2002, Li *et al.* 2002b, Ganz & Vita 2003, Dickson & Gotlieb 2004, Förstermann & Münzel 2006, Landmesser & Drexler 2007, Rossi *et al.* 2008). This brief, non-exhaustive review focuses on the imbalance between opposing endothelium-derived mediators, in particular NO and EDCFs, and its role in the genesis of vascular disease.

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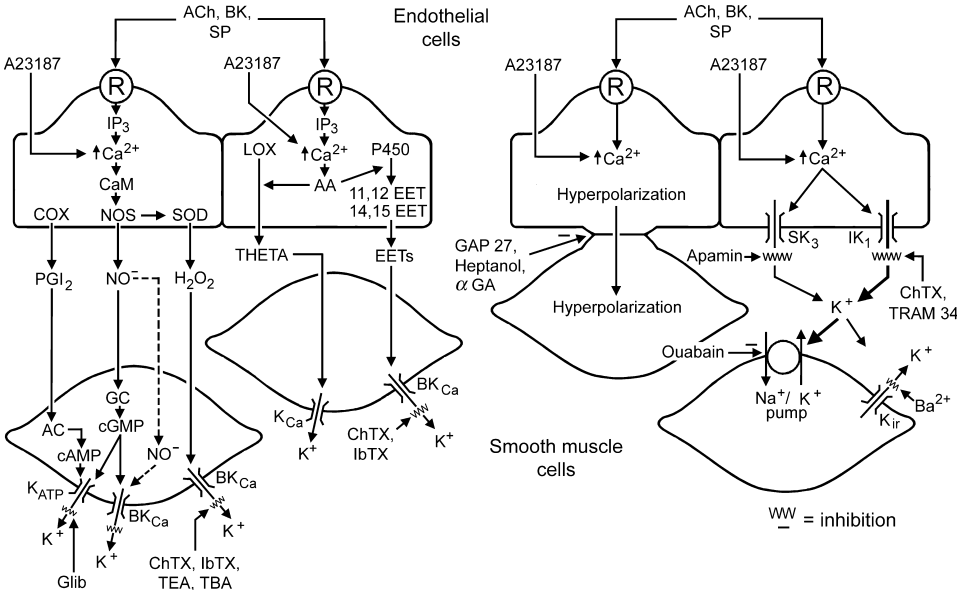

Figure 3 Multiplicity of mechanisms leading to endothelium-dependent hyperpolarization. Substances such as acetylcholine (Ach), bradykinin (BK) and substance P (SP), through the activation of M_3-muscarinic, B_2-bradykinin and NK_1-neuroknin receptor subtypes, respectively, and agents that increase intracellular calcium, such as the calcium ionophore A23187, release endothelium-derived hyperpolarizing factors. CaM, calmodulin; COX, cyclooxygenase; EET, epoxyeicosatrienoic acid; IP_3, inositol trisphosphate; GC, guanylate cyclase; NAPE, *N*-acylphosphaticylethanolamine; Hyperol., hyperpolarization; 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 (CB_1). Glibenclamide (Glib) is a selective inhibitor of ATP-sensitive potassium channels (K^+_{ATP}). Tetraethylammonium (TEA) and tetrabutylammonium (TBA) are nonspecific 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^+_{Ca2+}). Iberiotoxin (IBX) is a specific inhibitor of large conductance K^+_{Ca2+}. Charybdotoxin (CTX) is an inhibitor of large conductance K^+_{Ca2+}, intermediate conductance K^+_{Ca2+}(IK^+_{Ca2+}) and voltage-dependent potassium channels. Apamin is a specific inhibitor of small conductance K^+_{Ca2+} (SK^+_{Ca2+}). Barium (Ba^{2+}), in the micromolar range, is a specific inhibitor of the inward rectifier potassium channel (K_{ir}). GAP 27 (an 11-amino acid peptide possessing conserved sequence homology to a portion of the second extracellular loop of connexin), 18α-glycyrrhetinic acid (αGA) and heptanol are gap junction uncouplers.

Nitric oxide

Protector of the vascular wall

As such, the endothelium-dependent relaxation to acetylcholine 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. A number of more physiological stimuli {physical forces, circulating hormones [catecholamines, vasopressin, aldosterone], platelet products [serotonin, adenosine diphosphate (ADP)], autacoids [histamine, bradykinin], prostaglandin E_4, thrombin} share with acetylcholine the ability to elicit endothelium-dependent changes in the tone of the underlying smooth muscle (Fig. 1) (see Vanhoutte *et al.* 1986, Lüscher & Vanhoutte 1990, Pearson & Vanhoutte 1993, Stähli *et al.* 2006, Hristovska *et al.* 2007, Levine *et al.* 2007, Touyz 2007, Tang *et al.* 2008). NO plays a key role in the protection exerted by the endothelium against coronary disease. It is produced by the Ca^{2+}-dependent constitutive isoform of NO synthase (eNOS, NOS III) (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). 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 (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, Lüscher *et al.* 1993, Li *et al.*

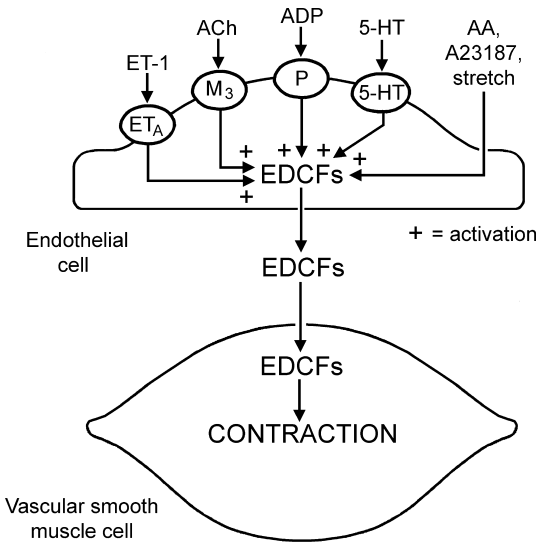

Figure 4 Under certain conditions, the endothelial cells, when activated by neurohumoral mediators, subjected to sudden stretch or exposed to the Ca^{2+} ionophore A23187, release a vasoconstrictor substance(s), termed endothelium-derived contracting factor (EDCF(s)), which diffuses to the underlying 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.

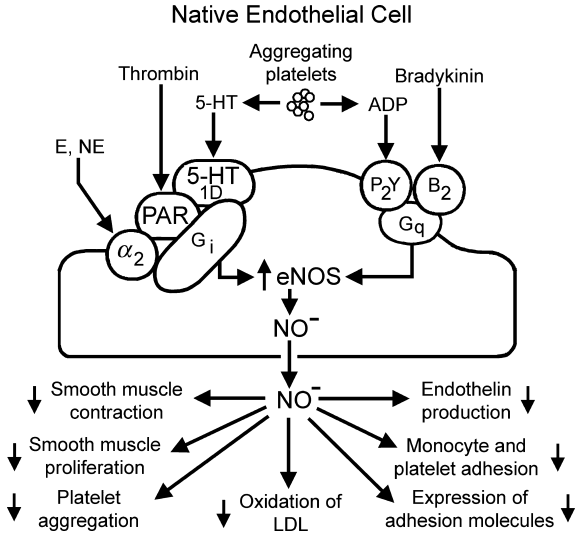

Figure 5 Postulated G-protein-mediated signal transduction processes in a normal, native endothelial cell. Activation of the cell causes the release of nitric oxide (NO), which has important protective effects in the vascular wall. 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G,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

2002b, Vallance 2003, Cooke 2004, Voetsch *et al.* 2004, Félétou & Vanhoutte 2006a,b,c).

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,b, Derkach *et al.* 2000, Motley *et al.* 2007, Touyz 2007) and *in vivo* (Shimokawa & Vanhoutte 1991). Serotonin (5-hydroxytryptamine, 5HT) and 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 Gi-proteins, while the P_{2y}-purinoceptors are linked to the enzyme by Gq-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 (see Vanhoutte 1988, 1996, 1997, 2002, Félétou and Vanhoutte 2006b). Thus, if platelet aggregation occurs in a coronary artery with a healthy endothelium the release by the platelets of serotonin (and ADP) and the local production of thrombin will stimulate the endothelial cells with the resulting release of 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 interrupted by injury, the aggregating platelets can approach 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 the most prominent in the coronary and cerebral circulations.

Modulation of the protective role of nitric oxide

The ability of the endothelium to release NO can be up-regulated or down-regulated in the intact organism by a number of chronic factors.

Up-regulation. Shear stress: Both acute and chronic increases in flow, and the resulting increasing force of shearing (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) (Rubanyi *et al.* 1986, Miller & Vanhoutte 1988, Davies 1995, Davis *et al.* 2001, Stepp

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et al. 2001, Busse & Fleming 2003, Bellien *et al.* 2006, Spier *et al.* 2007, Yan *et al.* 2007). This immediate effect of an increase in shear stress on the release of NO explains flow-mediated dilatation, a phenomenon often used to estimate the functional state of the endothelium in humans. In the coronary circulation, the effect of shear stress involves the local production of the autacoid bradykinin that stimulates the release of NO through a Gq-dependent mechanism (Fig. 6) (Flavahan *et al.* 1989, Mombouli & Vanhoutte 1991, 1995, Shimokawa *et al.* 1991, Roves *et al.* 1995). The chronic effect of shear stress is due to an up-regulation of eNOS and a greater 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, Hambrecht *et al.* 2003, Suvorava *et al.* 2004, Watts *et al.* 2004, Lauer *et al.* 2005, Gertz *et al.* 2006, Rakobowchuk *et al.* 2008).

Oestrogens and gender. Although ovariectomy does not alter or even increase the mRNA expression and the presence of eNOS (Wassmann *et al.* 2001, Okano *et al.* 2006), the reintroduction of physiological levels of oestrogens in ovariectomized animals augments endothelium-dependent relaxations to muscarinic agonists (Gisclard *et al.* 1988, Wassmann *et al.* 2001, Santos *et al.* 2004, Scott *et al.* 2007) and accelerates endothelial healing after injury (Filipe *et al.* 2008). The potentiating effect of oestrogens on endothelium-dependent relaxations involves both genomic (Fig. 2) and non-genomic effects (see Tostes *et al.* 2003, Keung *et al.* 2005, Miller & Duckles 2008). It depends presumably both on a reduction in oxidative stress leading to an increased bioavailability of the endothelium-derived mediator 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,b, Scott *et al.* 2007). In the intact organism, a reduced production of the endogenous inhibitor of eNOS, asymmetric dimethyl arginine (ADMA) may contribute (Filser 2005, Monsalve *et al.* 2007). Phytooestrogens and selective oestrogen receptor modulators also 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 seen only with stimuli that activate Gi-coupled receptors on the endothelial cells and is counteracted by the chronic administration of progesterone (Miller & Vanhoutte 1991). It is likely that this 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 (Kauser & Rubanyi 1995, Kähönen *et al.* 1998, Dantas *et al.* 2004) and thus why women are protected against coronary disease, at least until the age of menopause. The opposing effects of oestrogens and progesterone could explain why hormone replacement therapy has not always had the expected beneficial effect on the occurrence of cardiovascular events.

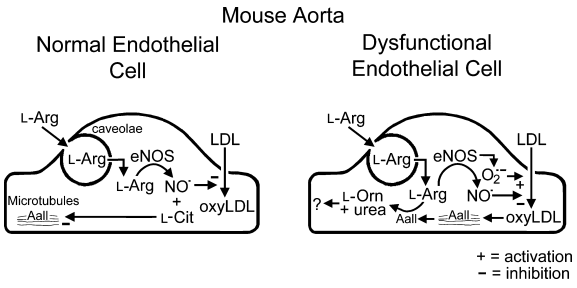

Figure 6 Model of endothelial dysfunction in the hypercholesterolaemic mouse. *Left*: in the normal mouse aortic endothelium, L-arginine (L-Arg) is transformed by eNOS to NO, which exerts its well-documented beneficial effects (most are not shown for the sake of clarity), including inhibition of the oxidation of LDLs to oxy-LDL. 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 oxy-LDL 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 oxy-LDL. 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 (modified from Vanhoutte 2008).

Insulin. Insulin facilitates NO-dependent vasodilatations *in vivo* (Steinberg *et al.* 1994, Taddei *et al.* 1995b, Lembo *et al.* 1997). It enhances the expression of eNOS in native endothelial cells *in vitro* (Fisslthaler *et al.* 2003).

Adiponectin. Adiponectin improves endothelial function and protects the endothelium by promoting eNOS activity and the bioavailability of NO (Chen *et al.* 2003, Hattori *et al.* 2003, Tan *et al.* 2004, Li *et al.* 2007b, Wang & Scherer 2008, Zhu *et al.* 2008).

Other hormones. In postmenopausal women, testosterone appears to potentiate endothelium-dependent vasodilatation (Montalcini *et al.* 2007). Thyroid hormone up-regulates eNOS and augments the endothelial production of NO in the animal (Spooner *et al.* 2004).

Adrenalectomy augments the expression of eNOS (Li *et al.* 2007a) and aldosterone acutely augments NO-dependent relaxations by a non-genomic action (Uhrenholt *et al.* 2003, 2004, Skott *et al.* 2006, Nietlispach *et al.* 2007). Glucagon-like peptide-1 enhances the vasodilator response to acetylcholine (Basu *et al.* 2007).

Diet. The chronic intake of ω_3-unsaturated fatty acids potentiates the endothelium-dependent relaxations of coronary arteries to aggregating platelets and other stimuli and have antiatherogenic properties (Shimokawa *et al.* 1987, 1988a,b, Shimokawa & Vanhoutte 1989a, Shepherd & Vanhoutte 1991, von Schacky & Harris 2007, Sekikawa *et al.* 2008, Sena *et al.* 2008). The same holds true for the intake of flavonoids (Machha & Mustafa 2005, Machha *et al.* 2007, Xu *et al.* 2007) and other polyphenols, whether present in red wine (in particular resveratrol) (Stockley 1998, Leikert *et al.* 2002, Wallerath *et al.* 2002, da Luz & Coimbra 2004, Dell'Agli *et al.* 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), in green tea (Kuriyama *et al.* 2006, Alexopoulos *et al.* 2008), 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).

Arginine. Although the acute administration of L-arginine can favour endothelium-dependent responses in humans (e.g. Bode-Böger *et al.* 1996, Taddei *et al.* 1997b, Perticone *et al.* 2005), its chronic supplementation offers no therapeutic benefit in patients with vascular disease (Wilson *et al.* 2007), reinforcing the early suspicion (Schini & Vanhoutte 1991a,b) that the semi-essential amino acid is rarely a limiting factor for the endothelial production of NO. An exception may be when the endothelial arginases, which compete with eNOS for this substrate, are more active (Fig. 6) (Ming *et al.* 2004, Brandes 2006, Ryoo *et al.* 2006, 2008, Holowatz & Kenney 2007, Katusic 2007, Santhanam *et al.* 2007, Romero *et al.* 2008, Vanhoutte 2008).

Down-regulation. Oxygen-derived free radicals: Several enzymes in the endothelial cells can produce superoxide anions (Fig. 7). They include NADPH oxidase, xanthine oxidase, cyclooxygenase and eNOS itself, when it is uncoupled by lack of substrate (L-arginine) or shortage of the essential co-factor tetrahydrobiopterin (BH4) (see Kojda & Harrison 1999, Stuehr *et al.* 2001, Fleming *et al.* 2005). Superoxide anions can be dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) which can act as an EDHF and contribute to endothelium-dependent relaxations (Fig. 2) (Matoba *et al.* 2000, Morikawa *et al.* 2003, Shimokawa & Matoba 2004; see Félétou & Vanhoutte 2006a,b,c, 2007), or be broken down by catalase. However, superoxide anions also 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 (see Kojda & Harrison 1999). Hence, increases in oxidative stress have been consistently associated with reduced endothelium-dependent relaxations, and antioxidants shown to acutely improve such responses *in vitro* and *in vivo* both in animals (e.g. Aubin *et al.* 2006, Liu *et al.* 2007) and humans (e.g. Kanani *et al.* 1999, Taddei

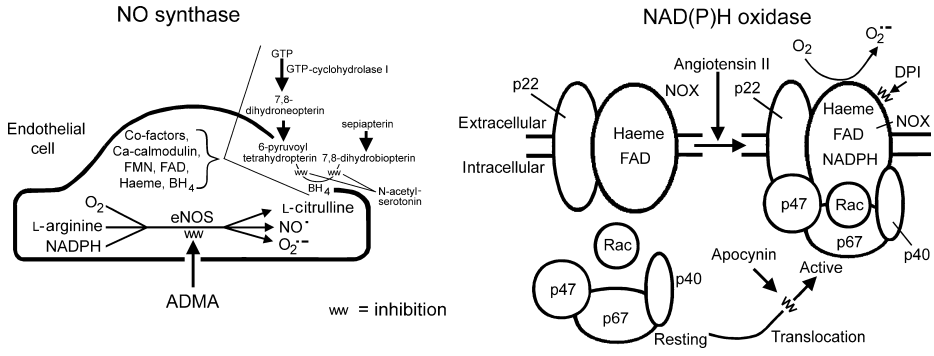

Figure 7 Two major contributors of reactive oxygen species in the vascular wall. *Left*: L-arginine-endothelial NOS (eNOS) pathway. Synthetic pathway of tetrahydrobiopterin (BH_4), an essential cofactor, is also shown and some of the most common inhibitors of NOS, analogues of L-arginine, are indicated. 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 nonspecific inhibitor of NOX (from Félétou and Vanhoutte 2006b. By permission of the American Physiological Society).

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et al. 2001, Holowatz & Kenney 2007). However, the therapeutic relevance of these findings is questionable as chronic treatment with antioxidants usually fails to improve endothelial function in people (e.g. Duffy *et al.* 2001, Pellegrini *et al.* 2004), with maybe the exception of the chronic administration of low doses of folic acid (Moat *et al.* 2006).

Hormones. Long-term exposure to aldosterone has a detrimental effect on NO-dependent relaxations, presumably by reducing the production of the essential cofactor for eNOS, tetrahydrobiopterin and increasing oxidative stress (Mitchell *et al.* 2004, Hashikabe *et al.* 2006, Nagata *et al.* 2006, Skott *et al.* 2006, Nietlispach *et al.* 2007, Sartorio *et al.* 2007). Melatonin also inhibits the endothelial formation of NO (Silva *et al.* 2007). 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* (see Moritoki *et al.* 1986, Hongo *et al.* 1988, Koga *et al.* 1988, Charpie *et al.* 1994, Kung & Lüscher 1995, Davidge *et al.* 1996, Chauhan *et al.* 1996, Taddei *et al.* 1997b, 2001, 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). This is due to an increased activity of arginase, competing with eNOS for the common substrate arginine (Katusic 2007, Santhanam *et al.* 2007), an 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), a reduced expression/presence of eNOS (Challah *et al.* 1997, Chou *et al.* 1998, Csiszar *et al.* 2002), a reduced activity of the enzyme (Cernadas *et al.* 1998) and ultimately a lesser release of NO (Tschudi *et al.* 1996a). In addition, the expression of soluble guanylyl cyclase in ageing vascular smooth muscle is reduced (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 EDCF).

Smoking and environment. Active and passive smoking blunt endothelium-dependent vasodilatations. This appears to be due to an action of nicotine causing a greater formation of ADMA and to an increased production of oxygen-derived free radicals, both resulting in a lesser availability of NO (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). Chronic exposure to air pollution decreases endothelium-dependent vasodilatations (Briet *et al.* 2007).

Homocysteinaemia. Increased levels of homocysteine impair eNOS-dependent relaxations/vasodilatations both *in vitro* and *in vivo*, presumably by increasing oxidative stress (e.g. Bellamy *et al.* 1998, Chambers *et al.* 1999, Kanani *et al.* 1999, Lang *et al.* 2000, Hanratty *et al.* 2001, Liu *et al.* 2007, Looft-Wilson *et al.* 2008).

Hypercholesterolaemia. Both in animals and in humans, hypercholesterolemia reduces 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). This is explained best by an increased oxidative stress leading to a reduced bioavailability of NO, an impairment of the turnover rate of eNOS and an increased presence of ADMA (Bode-Böger *et al.* 1996, Böger & Bode-Böger 2001, Böger *et al.* 2004, August *et al.* 2006, Palm *et al.* 2007).

Obesity. Obese animals and humans exhibit reduced responses to endothelium-dependent vasodilators (Karagiannis *et al.* 2003, Van Guilder *et al.* 2006, 2008, Bouvet *et al.* 2007, Kagota *et al.* 2007). A major reason for the blunted endothelium-dependent relaxation is the production of EDCF (see section EDCF). 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).

Sleep apnoea. Intermittent hypoxia, as occurring with obstructive sleep apnoea reduces endothelium-dependent responsiveness (Budhiraja *et al.* 2007).

Hallmark of disease

Hypertension. Endothelium-dependent relaxations are reduced in isolated arteries from different animal models of hypertension (e.g. Lüscher *et al.* 1987a,b, 1992, Hongo *et al.* 1988, Kung & Lüscher 1995, Vanhoutte & Boulanger 1995, Tschudi *et al.* 1996b, Vanhoutte 1996, Shimokawa & Vanhoutte 1997, Zhou *et al.* 1999). Likewise, the response to endothelium-dependent vasodilators is blunted in hypertensive humans (e.g. Taddei *et al.* 1995a, 1997a, 2001, Perticone *et al.* 2005). This blunting can be corrected by an appropriate treatment both in animals and in people (Lüscher *et al.* 1987b, Hutri-Kahonen *et al.* 1997, Taddei *et al.* 1998, Benndorf *et al.* 2007, Naya *et al.* 2007). It probably reflects the premature ageing of the vasculature exposed chronically to the increased arterial blood pressure (Taddei *et al.* 1997b). In essential hypertension, the reduction in response to endothelium-dependent stimuli *in vivo* may be due in part to higher circulating levels of ADMA (Perticone *et al.* 2005). In spontaneously hypertensive rats (SHR), the blunting of endothelium-dependent relaxations/

vasodilatations is due mainly to the concomitant release of endothelium-derived vasoconstrictor prostanoids (see section Hallmark of vascular disease) rather than to a reduced release of NO (Lüscher & Vanhoutte 1986, Lüscher *et al.* 1987d, Koga *et al.* 1988, Yasuro *et al.* 1999) 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).

Diabetes. Insulin resistance and diabetes cause an impairment of arterial endothelium-dependent relaxations in animals and humans, presumably due to the chronic exposure to hyperglycaemia (see 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, Schäfer *et al.* 2008). 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: (1) reduced bioavailability of tetrahydrobiopterin and uncoupling of eNOS (Guzik *et al.* 2002, Pannirselvam *et al.* 2002, Alp *et al.* 2003, Cai *et al.* 2005); (2) increased activity of arginase competing with eNOS for the common substrate, arginine (Ming *et al.* 2004, Ryoo *et al.* 2006, 2008, Katusic 2007, Lüscher & Steffel 2008, Romero *et al.* 2008, Vanhoutte 2008); (3) elevated levels of the endogenous inhibitor of eNOS ADMA (Lin *et al.* 2002, Xiong *et al.* 2003); (4) 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, Luscher & Steffel 2008, Schäfer *et al.* 2008); (5) quenching of NO by advanced glycosylation products (Bucala *et al.* 1991, Yin & Xiong 2005, Gao *et al.* 2008); (6) reduced presence of apelin (Grisk 2007, Zhong *et al.* 2007); and (7) abnormal responsiveness of vascular smooth muscle (Lu *et al.* 2005, Lesniewski *et al.* 2008, Shi *et al.* 2008). In addition to a reduced bioavailability of NO, the production of vasoconstrictor prostanoids contributes importantly to the endothelial dysfunction of diabetes (see section Hallmark of vascular disease).

Coronary disease. Individuals at increased risk of coronary heart disease are characterized by impaired peripheral dilatations in response to acetylcholine (Ijzerman *et al.* 2003). Also in the coronary circulation, endothelial dysfunction is a characteristic of the disease. (e.g. Ludmer *et al.* 1986, Hodgson & Marshall 1989, Shimokawa & Vanhoutte 1997, Vanhoutte *et al.* 1997, Lavi *et al.* 2008). 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).

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 under-perfusion of the tissues and the leading to down-regulation 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). An impairment of the ability of the vascular smooth muscle cells to relax contributes to the blunting of the endothelium-dependent responsiveness (Gill *et al.* 2007). The degree of impairment of endothelium-dependent vasodilatations predicts the outcome in patients with chronic heart failure (Meyer *et al.* 2005).

Pulmonary hypertension. Chronic hypoxia resulting in pulmonary hypertension results in 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, a phenomenon exacerbated by the genetic deletion of bone morphogenetic protein receptors (Fresquet *et al.* 2006, Frank *et al.* 2008). 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 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 bloodstream. 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

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1997, Hibbert *et al.* 2003, Sata 2003, Dimmeler & Zeiher 2004, Lamping 2007, Filipe *et al.* 2008, Zampetaki *et al.* 2008).

Regenerated endothelial cells are dysfunctional (Fig. 8). This conclusion is based on experiments performed on porcine coronary arteries (Shimokawa *et al.* 1989, 1991, Eto *et al.* 2005). Thus, 1 month after *in vivo* balloon denudation of the endothelium of part of the artery, despite total relining of the endothelial surface, rings covered with regenerated endothelium exhibited a marked blunting of the relaxations to aggregating platelets, serotonin or thrombin and the remaining response is no longer inhibited by pertussis toxin. By contrast, relaxations evoked by ADP and bradykinin, which both depend on the Gq-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 observations implied a selective dysfunction of the Gi-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 (Shimokawa & Vanhoutte 1989a,c). 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 on 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).

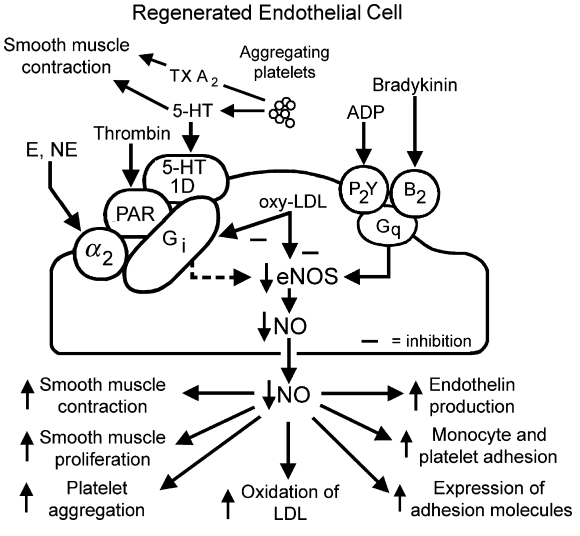

Figure 8 Effects of oxidized low-density lipoproteins (oxy-LDL) in a regenerated endothelial cell, resulting in the reduced release of nitric oxide (NO). 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G, coupling proteins.

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 NADPH oxidase), took up more modified low-density lipoprotein cholesterol (LDL) and generated more oxidized LDL (oxy-LDL). By contrast, the presence of Gi-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 oxy-LDL 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 prompted the assumption that an augmented presence of oxy-LDL contributes to the selective loss in Gi-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 oxy-LDL which play a central role in the atherosclerotic process (Fig. 9) (Stocker & Keaney

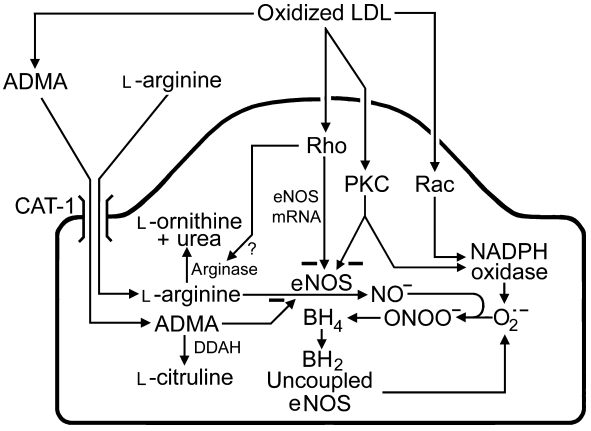

Figure 9 Mechanisms of oxy-LDL-induced impairment of endothelial NO production. The NO synthase (NOS) uses L-arginine to generate NO. NO production could be attenuated in the presence of oxy-LDL by interfering with the supply of L-arginine to the enzyme through endogenous competitive inhibitors such as asymmetrical dimethyl-L-arginine (ADMA) as well as degradation of arginine through arginase. NOS expression and specific activity are decreased by oxy-LDL through RhoA and PKC. NO bioavailability is reduced by an oxy-LDL-mediated activation of NADPH oxidase, which leads to superoxide anion (O_2^-) formation. This process facilitates the generation of peroxynitrite ($ONOO^-$), which subsequently oxidizes tetrahydrobiopterin (BH_4) of NOS, leading to NOS uncoupling. Uncoupled NOS itself produces O_2^-, further promoting the process of BH_4 oxidation. Rho, member of the Rho protein family (either RhoA or Rac) (modified from Brandes 2006).

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 oxy-LDL, may accelerate or contribute to the atherosclerotic process. These include: (1) emergence of fatty acid-binding proteins (Furuhashi *et al.* 2007, Lee *et al.* 2007, Furuhashi & Hotamisligil 2008, Hoo *et al.* 2008); (2) circulating chemokines (Ardigo *et al.* 2007); (3) inhibition of the proteosome (Herrmann *et al.* 2007); (4) presence of growth-related oncogene-α (Bechara *et al.* 2007); and (5) insufficiency of the Paraoxonase-1 gene (Guns *et al.* 2008).

Regardless of the cause 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).

EDCF

The villains: endothelium-derived vasoconstrictor prostanoids

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 are responsible for these endothelium-dependent increases in vasomotor tone (Rubanyi & Vanhoutte 1985, Iqbal & Vanhoutte 1988, Yang *et al.* 2003). Although endothelial cells can produce endothelin-1 (Yanagisawa *et al.* 1988, Yanagisawa & Masaki 1989, Schini & Vanhoutte 1991c, Vanhoutte 1993a,b, Rubanyi & Polokoff 1994, Böhm & Pernow 2007, Kirkby *et al.* 2008) and other non-prostanoid vasoconstrictor substances (Dhein *et al.* 1997, Saifeddine *et al.* 1998; Jankowski *et al.* 2005), the available evidence strongly suggests that that vasoconstrictor prostaglandins produced by cyclooxygenase in the endothelium explain most endothelium-dependent contractions (Fig. 10) (see Vanhoutte *et al.* 2005).

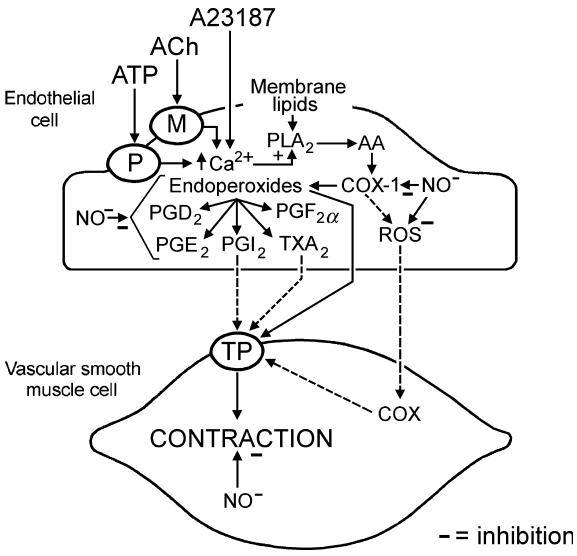

Figure 10 Endothelium-dependent contraction is likely to be composed of two components: generation of prostanoids and ROS. Each component depends on the activity of endothelial COX-1 and the stimulation of the TP receptors located on the smooth muscle to evoke contraction. In the spontaneously hypertensive rat aorta, there is an increased expression of COX-1 and EP3 receptors, increased release of calcium, ROS, endoperoxides and other prostanoids, which facilitates the greater occurrence of endothelium-dependent contraction in the hypertensive rat. The necessary increase in intracellular calcium can be triggered by receptor-dependent agonists, such as acetylcholine or ADP, or mimicked with calcium-increasing agents, such as the calcium ionophore A23187. The abnormal increase in intracellular ROS can be mimicked by the exogenous addition of H_2O_2 or the generation of extracellular ROS by incubation of xanthine with xanthine oxidase. AA, arachidonic acid; ACh, acetylcholine; ADP, adenosine diphosphate; H_2O_2, hydrogen peroxide; M, muscarinic receptors; P, purinergic receptors; PGD_2, prostaglandin 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.

EDCF-mediated responses. Endothelium-dependent, cyclooxygenase-dependent contractions 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, Wang *et al.* 2003, Vanhoutte *et al.* 2005).

Key role of endothelial cyclooxygenase. Early studies demonstrated that endothelium-dependent contractions are prevented by non-selective inhibitors of cyclooxygenase (Miller & Vanhoutte 1985, Lüscher &

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Vanhoutte 1986, Katusic *et al.* 1988), exemplifying the pivotal role of this enzyme in the phenomenon (see Vanhoutte *et al.* 2005). Bioassay studies indicate that the vasoconstrictor prostanoids involved are produced by the endothelial cyclooxygenase, rather than that of the vascular smooth muscle (Fig. 3) (Yang *et al.* 2003). Studies in arteries of the SHR using preferential and selective inhibitors of the two isoforms of the enzyme [constitutive cyclooxygenase-1 (COX-1) and inducible cyclooxygenase-2 (COX-2)], molecular biology experiments (Fig. 4) and studies with blood vessels of genetically modified mice concur to suggest that COX-1 is the major source of EDCF (Ge *et al.* 1995, Traupe *et al.* 2002a, Ospina *et al.* 2003, Wang *et al.* 2003, Yang *et al.* 2003, Tang *et al.* 2005a, Gluais *et al.* 2006). However, if endothelial COX-2 is induced, the prostanoids generated by this isoform also evoke endothelium-dependent contractions (Camacho *et al.* 1998, Zerrouk *et al.* 1998, Garcia-Cohen *et al.* 2000, Álvarez *et al.* 2005, Blanco-Rivero *et al.* 2005, Hirao *et al.* 2008, Ikeda *et al.* 2008, Shi & Vanhoutte 2008).

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 ADP (activating purinoceptors; Koga *et al.* 1989, Mombouli & Vanhoutte 1993). Endothelium-dependent contractions are less prominent in lower extracellular Ca^{2+}-concentration, are reduced by vitamin D derivatives, are triggered by calcium ionophores such as A23187, and are paralleled by an increase in endothelial cytosolic Ca^{2+}-concentration (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). These findings prompt the conclusion that an increased intracellular Ca^{2+}-concentration is the initial trigger for endothelium-dependent contractions, presumably by activating phospholipase A_2 which then makes arachidonic acid available to the endothelial cyclooxygenase setting in motion the release of EDCF.

When prostacyclin turns bad. Cyclooxygenase transforms arachidonic acid into endoperoxides which are released during endothelium-dependent contractions. As endoperoxides *per se* can activate vascular smooth muscle they are an EDCF (Ito *et al.* 1991, Asano *et al.* 1994, Ge *et al.* 1995, Vanhoutte *et al.* 2005, Hirao *et al.* 2008). Endoperoxides are converted 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, Norel 2007). The expression of the prostacyclin synthase gene is the most abundant in endothelial cells. 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 (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). However, in particular during EDCF-mediated responses to other agonists (ADP, A23187, endothelin-1, thrombin, nicotine), thromboxane A_2 contributes (Katusic *et al.* 1988, Shirahase *et al.* 1988, Auch-Schwelk & Vanhoutte 1992, Buzzard *et al.* 1993, Taddei & Vanhoutte 1993, Derkach *et al.* 2000, Gluais *et al.* 2006, 2007).

TP receptors, the effector. Cyclooxygenase-dependent, endothelium-dependent contractions are inhibited by antagonists of thromboxane-prostanoid (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).

Modulation of ECDF-mediated responses

Reduction in NO production. Inhibitors of NO synthases cause an immediate potentiation of EDCF-mediated responses (Auch-Schwelk *et al.* 1992, Yang *et al.* 2002, Paulis *et al.* 2008). 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/constrictions (Félétou *et al.* 2008, Michel *et al.* 2008a).

Facilitation by oxygen-derived free radicals. In some arteries, SOD, that does not permeate cells, abolishes endothelium-dependent contractions suggesting that superoxide anions act as an intercellular messenger which turns on the production of vasoconstrictor prostanoids by the vascular smooth muscle cells (Katusic & Vanhoutte 1989, Katusic 1996). In other blood vessels, however, SOD does not affect endothelium-dependent contractions while cell-permeable scavengers of superoxide anions variably depress the response (Auch-Schwelk *et al.* 1989, Yang *et al.* 2002, 2003, Tang & Vanhoutte 2008a). Acetylcholine and A23187 cause a burst of endothelial free radical production

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(Tang *et al.* 2007). As the burst is prevented by indomethacin, cyclooxygenase appears to be the main source of superoxide anions, and their production is not a primary event (Tang *et al.* 2007). The pharmacological data available indicate that, once produced, the free radicals amplify the EDCF-mediated response, presumably in part by stimulating cyclooxygenase of the endothelial cells but also possibly by activating that of the vascular smooth muscle (Auch-Schwelk *et al.* 1989, Garcia-Cohen *et al.* 2000, Yang *et al.* 2002, 2003, Wang *et al.* 2003, Álvarez *et al.* 2008), although the latter conclusion is hard to reconcile with their extremely short half-life (with the exception of H_2O_2). Thus it is unclear how the oxygen-derived free radicals may reach the vascular smooth muscle cells. Whether or not and how the myo-endothelial gap junctions play a role in this transition remains to be determined, despite the fact that gap junction inhibitors reduce EDCF-mediated responses (Tang & Vanhoutte 2008a). Obviously, the scavenging action of superoxide anions on NO, by reducing the bioavailability of the latter (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) will also favour the occurrence of endothelium-dependent contractions. The resulting combination of the two radicals into peroxynitrite leads to tyrosine nitration of prostacyclin synthase (Zou *et al.* 2002). This may result in a compensatory production of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ and thus in augmented endothelium-dependent contractions (Zou *et al.* 1999, Bachschmid *et al.* 2003, Gluais *et al.* 2005).

Oestrogens and gender. In arteries of ovariectomized animals, chronic treatment with oestrogens reduces the augmented production of vasoconstrictor prostanoids by endothelial COX-1 and reduces the augmented responsiveness of the TP receptors on 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.

Ageing. 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). Inhibitors of cyclooxygenase, given *in vivo* or *in vitro*, prevent or revert, respectively, the blunting of endothelium-dependent relaxations/vasodilatations due to ageing (Koga *et al.* 1988, 1989, Davidge *et al.* 1996, Wang *et al.* 2003, Bulckaen *et al.* 2008). TP receptor antagonists have a similar effect (Kung & Lüscher 1995, Davidge *et al.* 1996, Abeywardena *et al.* 2002). The age dependency of the response is explained best by an increased oxidative stress resulting in the up-regulation of COX-1 and/or the induction of COX-2 (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).

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 humans (Lüscher *et al.* 1987c, Taddei *et al.* 1995a, 1997a,b).

Obesity. 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 up-regulation of the expression of TP receptors, and the unleashed production of endothelin-1 (Gollasch 2002, Traupe *et al.* 2002a,b, Mundy *et al.* 2007, Xiang *et al.* 2008).

Hallmark of vascular disease

Hypertension. The endothelium-dependent relaxations to acetylcholine are blunted and the endothelium-dependent contractions to acetylcholine more pronounced in arteries of the SHR than in those of normotensive Wistar-Kyoto rats (WKY) (Fig. 11) (Lockette *et al.* 1986, Lüscher & Vanhoutte 1986, Lüscher *et al.* 1987b, Koga *et al.* 1989, Kähönen *et al.* 1998). These changes are prevented by inhibitors of cyclooxygenase and antagonists at TP receptors (Lüscher & Vanhoutte 1986, Koga *et al.* 1989, Kung & Lüscher 1995, Zhou *et al.* 1999, Yang *et al.* 2003) The increase in intracellular endothelial Ca^{2+}-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 hypertensive strains the expression/presence of COX-1 is

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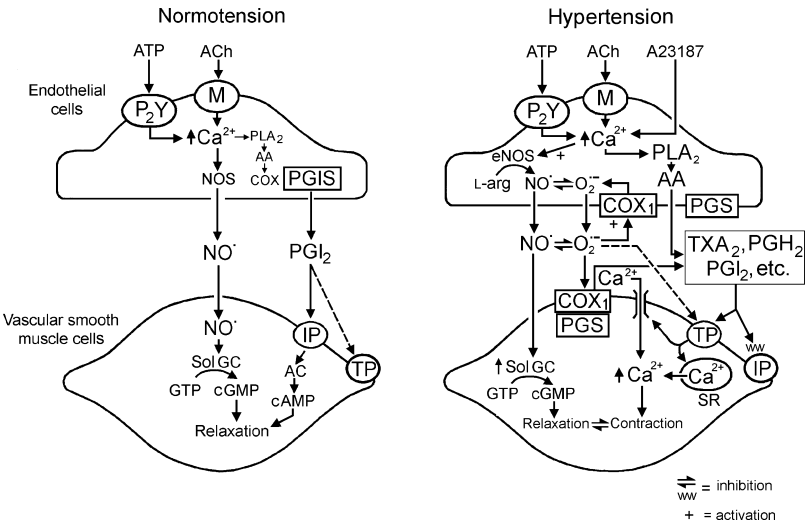

Figure 11 Endothelium-dependent effects of acetylcholine in rat aorta. *Left*: endothelium-dependent relaxations in normotensive rats. *Right*: cyclooxygenase-dependent, endothelium-dependent contractions to acetylcholine in SHR aorta. PGI_2, prostacyclin; R, receptor; IP, PGI_2 receptor; TP, TP receptor; PLA_2, phospholipase A_2; AA, arachidonic acid; COX_1, cyclooxygenase 1; S-18886 (terutroban), antagonist of TP receptors; M, muscarinic receptor, PGIS, prostacyclin synthase; PGH_2, endoperoxides; sGC, soluble guanylyl cyclase; AC, adenylyl cyclase; SR, sarcoplasmic reticulum; +, activation; −, inhibition; ?, unknown site of formation (from Félétou & Vanhoutte 2006b. By permission of the American Physiological Society).

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). Endothelium-dependent contractions are also facilitated by the fact that prostacyclin no longer causes relaxations in arteries of hypertensive animals (Rapoport & Williams 1996, 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 (Ge *et al.* 1999). Thus, it is not a consequence of premature ageing following the chronic exposure to an increased arterial blood pressure, and it constitutes one of the genetic platforms of the disease. 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).

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.* 1995a, 1997a,b, Monobe *et al.* 2001). This then suggests that EDCF-mediated responses also are part of the endothelial dysfunction of human hypertension.

Diabetes. The endothelium-dependent relaxations to acetylcholine are blunted in a number of arteries from diabetic animals (see 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 of both COX-1 and COX-2 (Tesfamariam *et al.* 1990, 1991, Shi *et al.* 2006, 2007a,b, 2008, Xu *et al.* 2006, Obrosova *et al.* 2007, Michel *et al.* 2008b, Shi & Vanhoutte 2008). In the case of diabetes, the production of reactive oxygen species (ROS) may play a more crucial role in triggering and amplifying EDCF-mediated responses (Shi *et al.* 2007b, 2008, Shi & Vanhoutte 2008).

Coronary disease. Aspirin and the TP receptor inhibitor terutroban improve endothelial function in patients with coronary disease, suggesting that endothelium-

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derived prostanoids contribute to the endothelial dysfunction resulting from the disease (Husain *et al.* 1998, Belhassen *et al.* 2003).

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. 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 oxy-LDL. 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 of endothelium-derived vasoconstrictor prostanoids (EDCF), in particular endoperoxides and prostacyclin. These prostanoids activate TP- receptors of the vascular smooth muscle leading to vasoconstriction which amplifies the degree of endothelial dysfunction. Whether or not the endothelial dysfunction caused by the imbalance between the production of NO and EDCFs can at least temporarily be compensated for in humans by EDHF-mediated responses (see Félétou & Vanhoutte 2006a,b, 2007) remains to be established.

Conflict of interest

There is no conflict of interest.

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