Endothelial Dysfunctionand 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- (e.g. by estrogens, exercise and dietary factors) and down-regulated (e.g. oxidative stress, smoking and oxidized low-density lipoproteins [oxyLDL]). 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 favors 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 nitric oxide is impaired (e.g. by oxidative stress, aging, spontaneous hypertension and diabetes). They contribute to the blunting of endothelium-dependent vasodilatations in aged subjects and essential hypertensive patients.

Short Title : NO, EDCF and Endothelial Dysfunction

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

1. Introduction

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) (Figure 1) [Furchgott & 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 (cyclic GMP) [see Ignarro et al., 1986; Furchgott & Vanhoutte, 1989; Lüscher & Vanhoutte 1990]. It is rapidly destroyed by superoxide anions [Rubanyi et al., 1986; Gryglewski et al., 1986]. These experimental facts led to the proposal [Furchgott, 1988; Ignarro et al., 1988a, 1988b] and the demonstration [Palmer et al., 1987, 1988, 1989; Moncada, 1997] that EDRF is nitric oxide (NO) (Figure 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 (Figure 3) [see Busse et al., 2002; Feletou & Vanhoutte 2006a, 2006b, 2007]. In addition, endothelial cells can release vasoconstrictor prostanoids (endothelium-derived contracting factors, EDCF) (Figure 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; Urakami-Harasawa et al., 1997; Feletou & 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; F□rstermann and Münzel, 2006; Landmesser & Drexler 2007, Ross 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.

2. Nitric Oxide:

2.1 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 neurons 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), 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(Figure 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) (Figure 2) [Marletta, 1989; Schini-Kerth & Vanhoutte, 1995; Moncada, 1997; Li et al., 2002a; Dudzinski et al., 2006; Feron and Balligand, 2006; O'Rourke et al., 2006]. NO not only prevents abnormal constriction (vasospasm) of the coronary arteries, which favors 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 (Figure 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; Feletou & Vanhoutte, 2006; Lüscher et al., 1993; Li et al., 2002b; Vallance, 2003; Cooke, 2004; Voetsch et al., 2004].

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., 1988; 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 (Figure 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; Feletou & 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 feed-back 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.

2.2. Modulation of the protective role of Nitric Oxide

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

2.2.1 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 (Figure 2) [Rubanyi et al., 1986; Miller & Vanhoutte, 1988; Davies, 1995; Davis et al., 2001; Stepp 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 (FMD), 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 (Figure 6) [Flavahan et al., 1989; Shimokawa et al., 1991; Mombouli & Vanhoutte, 1991, 1995; 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; Watts et al., 2004; Hambrecht et al., 2003; Suvorava et al., 2004; Lauer et al., 2005; Gertz et al., 2006; Rakobowchuk et al., 2008].

Estrogens and Gender: Although ovariectomy does not alter or even increase the mRNA expression and the presence of eNOS [Wassman et al., 2001; Okano et al., 2006], the reintroduction of physiological levels of estrogens in ovariectomized animals augments endothelium-dependent relaxations to muscarinic agonists [Gisclard et al., 1988; Wassman 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 estrogens on endothelium-dependent relaxations involves both genomic (Figure 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 [Wassman et al., 2001; Han et al., 2007; Li et al., 2007; Scott et al., 2007]. In the intact organism, a reduced production of the endogenous inhibitor of eNOS, asymmetric dimethyl arginine (ADMA) may contribute [Monsalve et al., 2007]. Phytoestrogens and selective estrogen receptor modulators (SERMs) 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 estrogens 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 estrogens 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 estrogens and progesterone could explain why hormone replacement therapy has not always had the expected beneficial effect on the occurrence of cardiovascular events.

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 post-menopausal 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 [Nietlispach et al., 2007; Uhrenholt et al., 2003, 2004; Skott et al., 2006]. Glucagon-like peptide-1 (GLP-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, 1988; Shimokawa & Vanhoutte, 1989a,; Shepherd & Vanhoutte, 1991; von Schacky & Harris, 2007; Sena et al., 2008; Sekikawa 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; Lefevre et al., 2007; Aubin et al., 2008; Chan et al.,

2008a, 2008b; 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., 2005, 2007a, 2007b] or in dark chocolate [Fischer et al., 2003; Engler et al., 2004;Grassi et al., 2005; Schroeter et al., 2006; Flemmer et al., 2007; Taubert et al., 2007].

Arginine: Although the acute administration of L-arginine can favor endothelium-dependent responses in humans [e.g. Bode-Boger 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] 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 (Figure 6) [Ming et al., 2004; Ryoo et al, 2006, 2008; Brandes, 2006; Holowatz & Kenney, 2007; Santhanam et al., 2007; Katusic, 2007; Romero et al., 2008; Vanhoutte 2008].

2.2.2. Down-regulation:

Oxygen-derived free radicals: Several enzymes in the endothelial cells can produce superoxide anions (Figure 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 (Figure 2) [Matoba et al., 2000; Morikawa et al., 2003; Shimokawa & Matoba, 2004; Takaki et al., 2008; see Feletou & Vanhoutte, 2006a, 2006c, 2007], or been broken down by catalase. However, superoxide anions also scavenge NO avidly with the resulting formation of peroxynitrite [Rubanyi & Vanhoutte 1986; Gryglewski et al., 1986; Auch-Schwelk et al., 1992; Cosentino et al., 1994; Tschudi et al., 1996a; De Lano 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 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].

Aging: 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 Vanhoutte, 2002; Moritoki et al., 1986; Koga et al., 1988; Charpie et al., 1994; Davidge et al., 1996; Hongo et al., 1988; Kung & Lüscher, 1995; Taddei et al., 1997b, 2001; Cernadas et al., 1998; Yasuro et al., 1999; Heymes et al., 2000; Csiszar et al., 2002, 2007; Subramian & 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 [Santhanam et al., 2007; Katusic, 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 aging vascular smooth muscle is reduced [Klöss et al., 2000]. However, an important part of the endothelial dysfunction with aging is due to the endothelial release of vasoconstrictor prostaglandins (see **3.2**).

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 [de Sousa et al., 2005; Michaud et al., 2006; Gamboa et al., 2007; Celermajer & Ng, 2008; Argacha et al., 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].

Homocysteinemia: 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].

Hypercholesterolemia: 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, 1989b; 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 et al., 2001, 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 **3**). 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 apnea: Intermittent hypoxia, as occurring with obstructive sleep apnea reduces endothelium-dependent responsiveness [Budhiraja et al., 2007].

2.3. Hallmark of disease

Hypertension: Endothelium-dependent relaxations are reduced in isolated arteries from different animal models of hypertension [e.g. Lüscher et al., 1987a, 1987b; Hongo et al., 1988; Vanhoutte & Boulanger, 1995; Kung & Lüscher, 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 aging 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 the spontaneously hypertensive rats, the blunting of endothelium-dependent relaxations/vasodilatations is due mainly to the concomitant release of endothelium-derived vasoconstrictor prostanoids (see **3.3**) 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 hyperglycemia [see De Vriese et al., 2000; Vallerjo et al., 2000; Cheng et al., 2001; Paennirselvam et al., 2002; Guzik et al., 2002; Inkster et al., 2002; Nassar et al., 2002; Kim et al., 2003; Kim et al., 2006; Shi et al., 2006; Eringa et al., 2007; Goel et al., 2007; Machha et al., 2007; Obrosova et al., 2007; Shi et al., 2007a; 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: a] reduced bioavailability of tetrahydrobiopterin and uncoupling of eNOS [Guzik et al., 2002; Pannirselvam et al., 2002; Alp et al., 2003; Cai et al., 2005]; b] 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]; c] elevated levels of the endogenous inhibitor of eNOSADMA [Lin et al., 2002; Xiong et al., 2003]; d] augmented production of

superoxide anions and thus scavenging of NO and increased presence of peroxynitrite [Cosentino et al., 1997; Mayhan & Patel, 1990; Graier et al., 1999; Maejima et al., 2001; Inkster et al., 2002; Pacher & Szabo, 2006; Duncan et al., 2007; Pannirselvam et al., 2002; Quijano et al., 2007; Gao et al., 2008; Lüscher & Steffel, 2008; Schäfer et al., 2008]; e] quenching of NO by advanced glycosylation products [Bucala et al., 1991; Yin & Xiong, 2005; Gao et al., 2008]; f] reduced presence of apelin [Zhong et al., 2007; Grisk, 2007]; and g] 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 **3.3**).

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; Vanhoutte et al., 1997; Shimokawa & Vanhoutte, 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; Ross 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; Ross 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].

2.4. 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 aging 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 neighboring cells, freed of contact inhibition, and circulating endothelial progenitor cells [Vanhoutte, 1997; Hibbert et al.,2003; Sata, 2003; Dimmeler and Zeiher, 2004; Lamping, 2007; Filipe et al., 2008; Zampetaki et al., 2008].

Regenerated endothelial cells are dysfunctional (Figure 8). This conclusion is based on experiments performed on porcine coronary arteries [Shimokawa et al., 1989d, 1991; Eto et al., 2005]. Thus, one 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 was no longer inhibited by pertussis toxin. By contrast, relaxations evoked by ADP and bradykinin, which both depend on the Gq-signaling 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

hypercholesterolemic diet which resulted in the occurrence of typical atherosclerotic lesions in the area of previous denudation [Shimokawa & Vanhoutte, 1989a, 1989c]. These observations prompt the conclusion that the selective dysfunction of regenerated endothelial cells is the first step allowing the atherosclerotic process.

To analyze 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]. 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 (oxyLDL). 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 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 assumption that an augmented presence of oxyLDL 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

[Figure 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 (Figure 9) [Stocker & Keany, 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 (Figure 6) [Ming et al., 2004; Ryoo et al., 2006, 2008; Brandes, 2006; 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: a] emergence of fatty acid-binding proteins [Lee et al., 2007; Furuhashi et al., 2007; Furuhashi & Hotamisligil, 2008; Hoo et al., 2008]; b] circulating chemokines [Ardigo et al., 2007]; c] inhibition of the proteosome [Herrman et al., 2007]; d] presence of growth-related oncogene-α [Bechara et al., 2007]; and e] insufficiency of the Paraoxonase -1 gene [Guns et al., 2008].

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; Aikawwa & Libby, 2004; Hansson, 2005; Barton et al., 2007].

3. EDCF:

3.1. 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 (Figure 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, 1991b; Vanhoutte, 1993; Rubanyi & Polokoff, 1994; Böhm & Pernow, 2007; Kirkby et al., 2008] and other non-prostanoid vasoconstrictor substances [Dhein et al., 1997; Saifedine 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 (Figure 10) [see Vanhoutte et al. 2005].

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; Vanhoutte et al., 2005; Derkach et al., 2000; Kauser & Rubanyi, 1995; Kähönen et al., 1998; Davidge & Zhang, 1998; Wang et al., 2003].

Key role of endothelial cyclooxygenase: Early studies already demonstrated that endothelium-dependent contractions are prevented by non-selective inhibitors of cyclooxygenase [Miller & Vanhoutte, 1985; Lüscher & 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 (Figure 3) [Yang et al., 2003]. Studies in arteries of the spontaneously hypertensive rat (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 (Figure 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; Alvarez et al., 2005; Blanco-Rivero et al., 2005; Hirao 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, 2007b, 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. Since 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].). 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; 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].

3.2. 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]. 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 favor the occurrence of EDCF-mediated contractions/constrictions [Feletou et al., 2008].

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]). 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 [Tang et al., 2007]. Since 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; Wang et al., 2003; Yang et al., 2002, 2003; Alvarez 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 [Rubanyi & Vanhoutte 1986; Gryglewski et al., 1986; Auch-Schwelk et al., 1992; Cosentino et al., 1994; Tschudi et al., 1996b; Touyz & Schiffrin, 2004; De Lano et al., 2006; Miyagawa et al., 2007; Macarthur et al., 2008] will also favor 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].

Estrogens and Gender: In arteries of ovariectomized animals, chronic treatment with estrogens 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]. Estrogens 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 estrogens on EDCF-mediated responses.

Aging: Endothelium-dependent contractions become more prominent with aging [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 aging [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; Tang & Vanhoutte, 2008b; Shi et al., 2008]. In addition, the expression of the prostacyclin synthase gene augments with age [Numaguchi et al., 1999]. Prostacyclin no longer evokes relaxations in arteries from aging 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 aging people, suggesting that the importance of EDCF-mediated responses also increases with age in the human [Lüscher et al., 1987c; Taddei et al., 1995a, 1997a, 1997b].

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 (Traupe et al., 2002a, b; Gollasch, 2002; Mundy et al., 2007; Xiang et al., 2008].

3.3. 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) (Figure 11) [Lockette et al., 1986;Lüscher & Vanhoutte, 1986; Lüscher et al. 1987b; Lüscher & Vanhoutte, 1986; Koga et al., 1989; Kähönen et al., 1998;]. These changes are prevented by inhibitors of cyclooxygenase and antagonists at TP-receptors [Lüscher & Vanhoutte, 1986; Kung & Lüscher, 1995; Zhou et al., 1999; Koga et al., 1989; 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 strain the expression/presence 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 aging 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; Rapoport & Williams 1996; Ge et al., 1995; 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 aging 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, 1997b; Monbe 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; Xu et al., 2006; Michel et al., 2008b; Shi et al., 2006, 2007a, 2007b, 2008; Obrosova et al., 2007; Shi & Vanhoutte, 2008]. In the case of diabetes, the production of 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-derived prostanoids contribute to the endothelial dysfunction resulting from the disease [Husain et al., 1998; Belhassen et al., 2003].

4. 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 leukocytes (macrophages). The endothelial mediator also prevents the proliferation of vascular smooth muscle cells and limits the formation of oxy LDL. Aging 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 Feletou & Vanhoutte 2006a, 2006b, 2007] remains to be established.

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Figure Legends:

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 (Norepinephrine); P, purinergic receptor; T, thrombin receptor; VEGF, vascular endothelial growth factorVP, 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 posttranslationally 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. +, indicates stimulation; -,

indicates inhibition; **?**, indicates those pathways in which the regulation is unknown. (Modified from O'Rourke et al. 2006)

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; $O_2^{\cdot-}$, 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 eleven-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.

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. Abbreviations: 5-HT, serotonin receptor; B, bradykinin receptor; P, purinoceptor; G, coupling proteins.

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 OxLDL. The byproduct 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 hypercholesterolemic mice, the accumulation of OxLDL 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 OxLDL. The latter also facilitates dissociation of eNOS from the caveolae and reduces the genomic expression of the enzyme, leading to further reduction of 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)

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 2006$_{b.}$ By permission of the American Physiological Society)

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

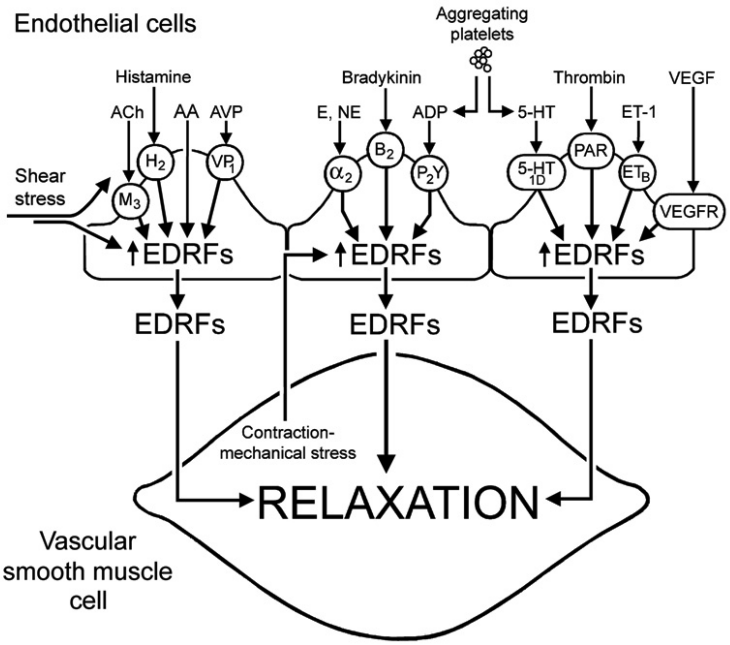
Figure 9. Mechanisms of oxLDL-induced impairment of endothelial NO production. The NO synthase (NOS) uses L-arginine to generate NO. NO production could be attenuated in the presence of oxLDL by interfering with the supply of L-arginine to the enzyme through endogenous competitive inhibitors such as asymmetrical dimethyl-L-arginine (ADMA) as well as degradation of arginine through arginase. NOS expression and specific activity are decreased by oxLDL through RhoA and PKC. NO bioavailability is reduced by an oxLDL-mediated activation of the 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)

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 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 = acetycholine; 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 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 and Vanhoutte 2006_b. By pemission of the American Physiological Society)

Figures



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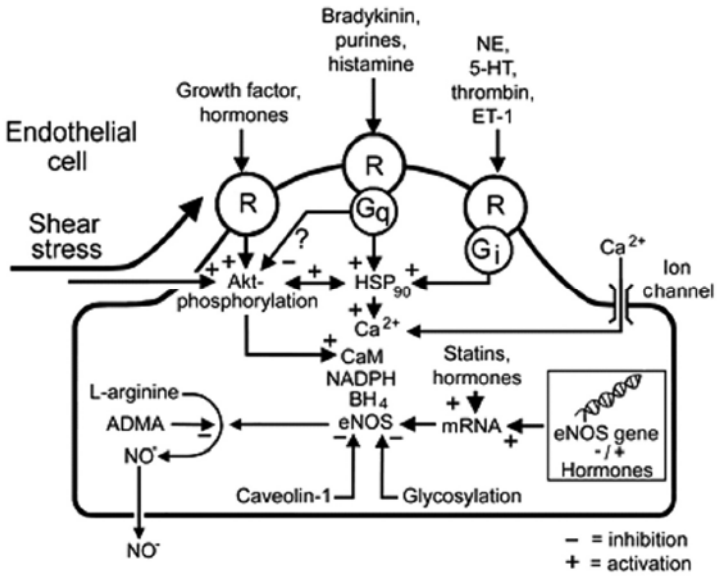

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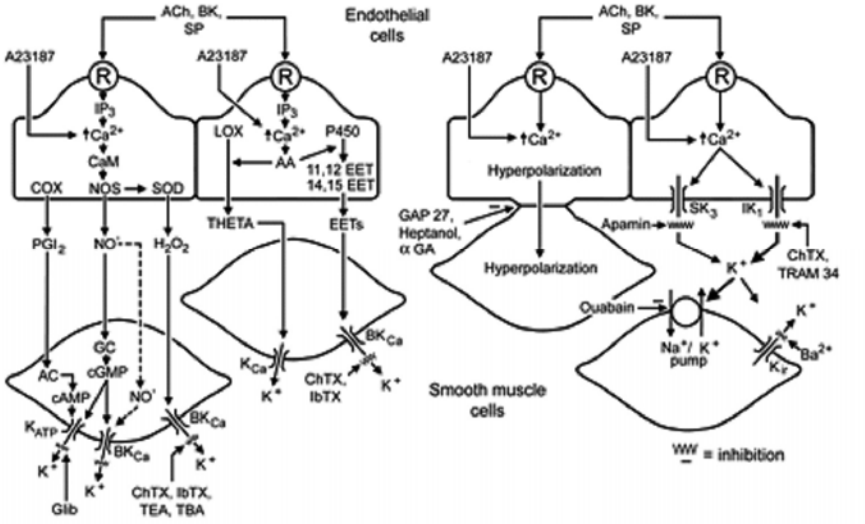

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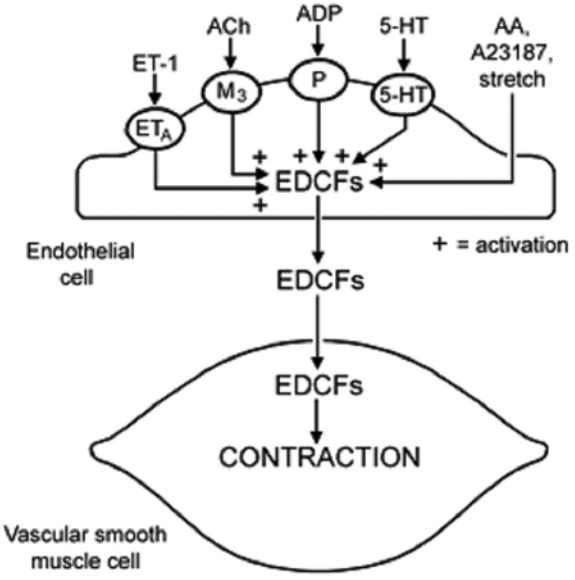

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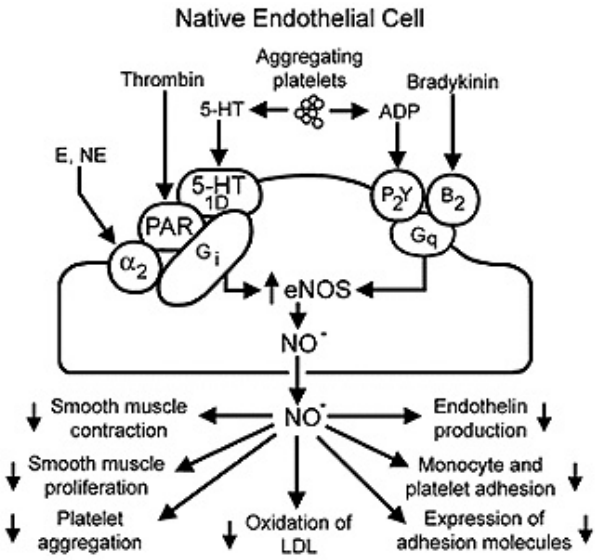
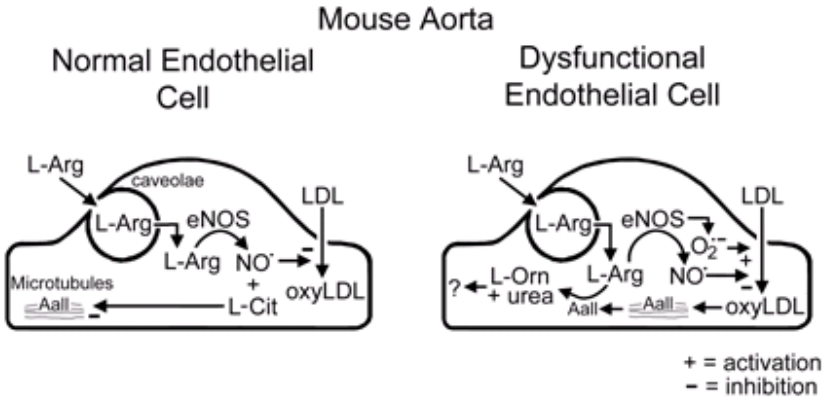

Native Endothelial Cell
Thrombin
Aggregating platelets
Bradykinin
5-HT
ADP
E, NE
5-HT 1D
PAR
P_2Y
B_2
G_q
G_i
α_2
eNOS
$NO^{\cdot}$
Smooth muscle contraction
Endothelin production
Smooth muscle proliferation
Monocyte and platelet adhesion
Platelet aggregation
Oxidation of LDL
Expression of adhesion molecules

Figure 5



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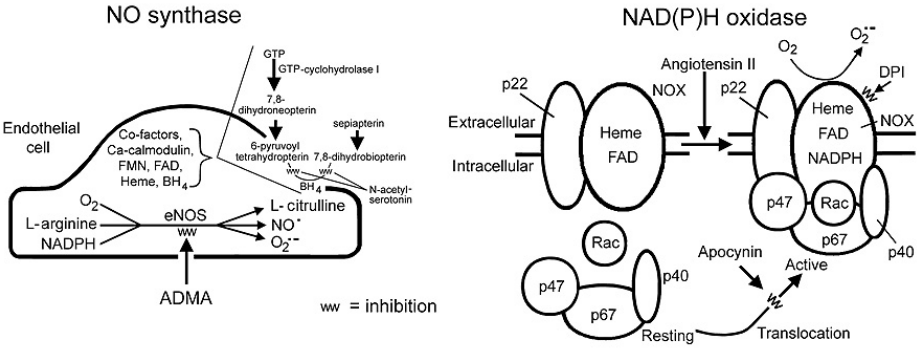

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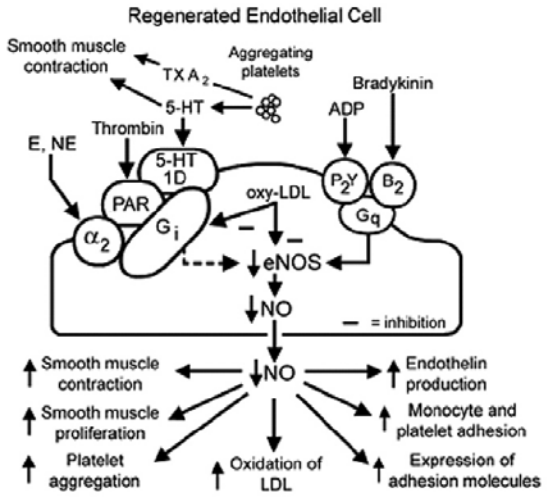

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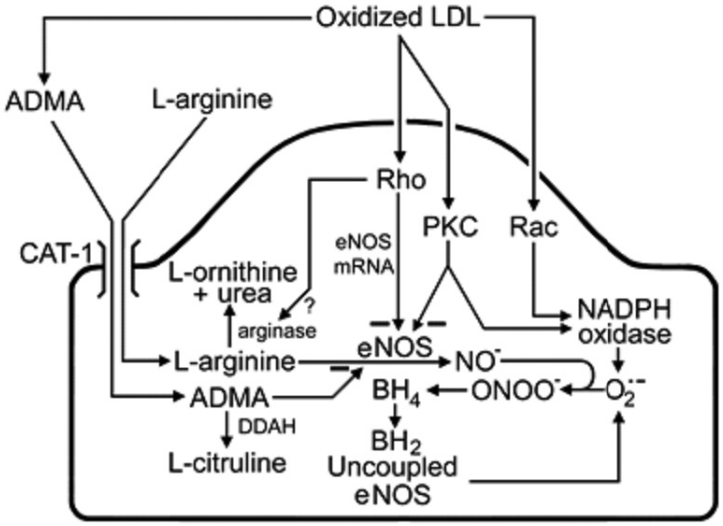

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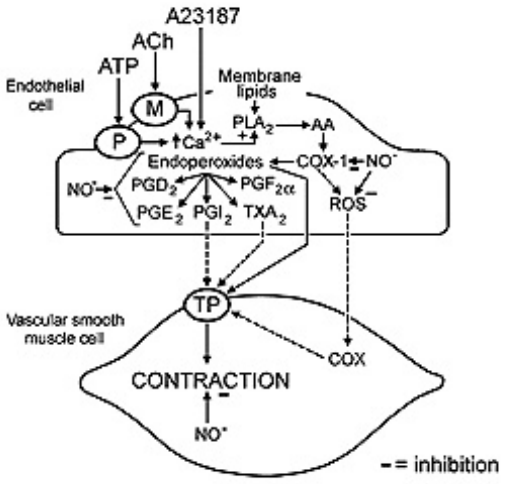

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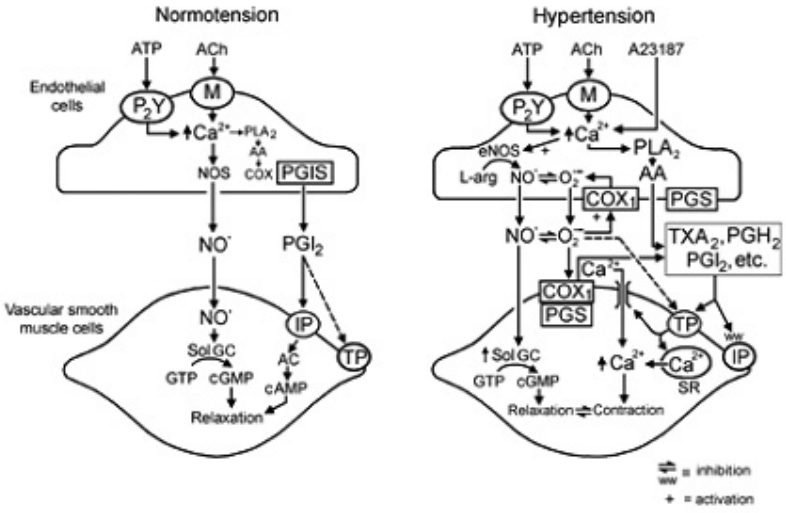

Figure 11