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Three decades of endothelium research

From the detection of nitric oxide to the everyday implementation of endothelial function measurements in cardiovascular diseases

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Summary

The endothelium is more than just a passive interface between the blood and the vessel wall. Since the pioneering discovery of nitric oxide as an important endothelium-derived vasorelaxing molecule three decades ago, vascular research has developed exponentially and remains fascinating for the entire research community. Endothelial dysfunction is a pathological condition characterized by an imbalance between vasodilatating and vasoconstricting substances. Most, if not all, cardiovascular risk factors have been attributed with endothelial dysfunction and their therapeutic modification with an improvement in vascular function. This overview aims to provide a glimpse into this fascinating research field with the emphasis on vasoactive substances and the assessment of endothelial function.

Key words: *endothelium; endothelial dysfunction; nitric oxide; vasoactive substances; flow-mediated vasodilatation*

Introduction

In the past the endothelium was believed to represent a simple semipermeable membrane covering the endoluminal part of all blood vessels. However, in recent years, abundant research on the endothelium and its function has brought to light its impressive and indeed indispensable physiological functions, especially in maintaining the homeostasis of vascular tone and structure. Loss of function of the endothelium not only makes the vessel prone to vasoconstriction, but also leads to atherothrombotic changes such as proliferation of vascular smooth muscle, expression of proinflammatory molecules and thrombosis.

Moreover, in humans endothelial dysfunction is one of the first detectable vascular alteration in the evolution of atherosclerosis, and its presence also correlates well with future cardiovascular events.

It is the aim of the present article to provide an overview of the physiological and pathophysiological function of the endothelium, its main vasoactive substances, and the possibilities of measuring and therapeutically influencing vascular function. The article is based upon the recently published overview article in a special issue on endothelium-dependent vasodilatation in honour of Robert Furchgott [1].

The vascular endothelium and its vasoactive substances

The endothelium represents the inner layer of the vessel wall. It is a continuous and smooth monolayer of cells providing a nonthrombogenic surface with highly selective permeability properties. In total, it represents a surface area of about 4000 to 7000 m^2. The endothelium controls vascular permeability and actively regulates the exchange of molecules in response to environmental and molecular signals (fig. 1) [2]. Moreover, healthy endothelial cells are crucial in the prevention of thrombotic events. A feature of note is that endothelial cells express antiplatelet and anticoagulant molecules [3], whereas dysfunctional cells make the vessel prone to thrombotic events with tissue factor playing an important role [4].

However, the endothelium is able to do much more; indeed, it is known to be a highly complex organ able to respond to a broad variety of endogenous and exogenous stimuli which also synthesizes and releases a vast amount of vasoactive substances.

Many endothelium-derived relaxing factors (EDRF) have been characterised chemically in recent years; most of

them are released in response to an increase in intracellular calcium. The most studied EDRF molecules are nitric oxide (NO), prostacyclin (PGI_2) and endothelial-derived hyperpolarisation factors (EDHF).

Furthermore, there are also important endothelial-derived constricting factors (EDCF), endothelin-1 (ET-1) representing the most potent molecule.

Given these important physiological (inter)actions of endothelial mediators, prompt repair of damaged or apoptotic cells by endothelial progenitor cells is essential. Thus, these cells are not only important for angiogenesis, but also prompt repair of defects in the endothelial lining of the vessel wall (for review see [5]).

Nitric oxide (NO)

Thirty years ago, Furchgott and Zawadzki demonstrated that endothelial cells produce a then unknown signaling molecule, which was at the time named endothelium-derived relaxing factor (EDRF) [6]. This molecule was shown to be able to relax vascular smooth muscles. Later on, Ignarro and Furchgott demonstrated that EDRF was indeed nitric oxide [7]. Since these discoveries a wealth of basic and clinical research has been triggered. For this seminal discovery Robert Furchgott and Louis Ignarro, together with Ferid Murad, were co-awarded the Nobel Prize in 1998.

In the course of further research, NO has not only been shown to have vasodilatory properties. Indeed, it also prevents platelet adhesion and aggregation, as well as leukocyte adhesion and migration into the arterial wall and inhibits smooth muscle cell proliferation, all key events in the development of atherosclerosis [8–13]. NO is a highly diffusible small molecule and is synthesised by NO synthase (NOS) from L-arginine. It is released by endothelial cells mainly in response to shear stress, but also by many other molecules such as acetylcholine, bradykinin, thrombin, and ADP among others, leading to a relaxation of vascular smooth muscle cells [6, 14–21].

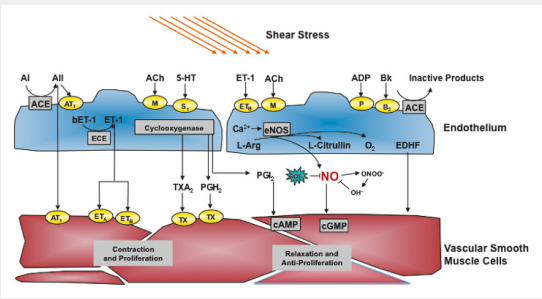

Figure 1

Endothelium-derived vasoactive substances.
Endothelial nitric oxide synthase is induced by shear stress and a variety of receptors and leads to a release of nitric oxide (NO), which exerts relaxation of vascular smooth muscle cells and other important effects such as antiproliferation and inhibition of thrombocyte aggregation and leukocyte adhesion. Other endothelium-derived relaxing factors including endothelium-derived hyperpolarisating factor (EDHF) and prostacyclin (PGI2) are also shown. ACE denotes angiotensin-converting enzyme, Ach, acetylcholine; AI, angiotensin I, AII, angiotensin II, AT1, angiotensin 1 receptor; Bk, bradykinin; COX, cyclooxygenase; ECE, ET-converting enzyme; EDHF, endothelium-derived hyperpolarizing factor; ET_A and ET_B, endothelin A and B receptors; ET-1, endothelin-1, L-Arg, L-arginine; PGH_2, prostaglandin H_2; ROS, reactive oxygen species; S_1, serotoninergic receptor; TH, thromboxane receptor; Thr, Thrombin; TXA_2, thromboxane; 5-HT, serotonin.

Prostacyclin

Prostacyclin (PGI_2) is another endothelium-derived relaxing factor which is released partly in response to shear stress [14, 22–24]. PGI_2 is synthesised by cyclooxygenase-1 (COX-1) from arachidonic acid [25] and increases cAMP in smooth muscle cells as well as in platelets. In contrast to NO, PGI_2 does not contribute to the maintenance of large conduit arteries' basal vascular tone [20]. Importantly, however, it has potent platelet inhibitory effects. In contrast to NO, which is released continuously by agonists [26], PGI_2 is released only in a transient manner [27]. PGI_2 facilitates the release of NO by endothelial cells [28] and vice versa, the action of PGI_2 in the vascular smooth muscle is also potentiated by NO and the half-life of the second messenger of prostacyclin is prolonged [29].

Endothelium-derived hyperpolarising factor(s)

Endothelium-derived hyperpolarising factors (EDHF) are molecules causing hyperpolarisation of smooth muscle cells. Their involvement in regulating vascular reactivity is defined as the endothelium-dependent response that persists in the presence of combined inhibition of NO and PGI_2. They might represent a compensatory mechanism for endothelium-dependent vasodilatation in the presence of reduced NO availability [30].

Studies have identified several molecules or mediators that might act as EDHF in different tissues and species [31]: among them K^+ [32], cytochrome P450 metabolites [33–35], lipoxygenase products [36], NO itself [37], reactive oxygen species (H_2O_2) [38], cyclic adenosine monophosphate [39], C-type natriuretic peptide [40], and electrical coupling through myoendothelial gap junctions [41, 42].

Endothelin

Some years after the detection of NO, the vasoconstrictor peptide endothelin (ET), which is also synthesised by vascular endothelial cells, was discovered [43, 44]. ET acts as a natural counterpart of NO [45]. Three isoformes of the peptide (ET-1, ET-2 and ET-3) exist, which are converted by the endothelin converting enzyme (ECE) from their precursors big endothelin originating from pre-proendothelin peptides cleaved by endopeptidases [46–50]. Similar to the expression of NO, there are also several factors modulating ET-1 production and release, among them shear stress, angiotensin II, thrombin, adrenaline, oxidised low-density lipoproteins and inflammatory cytokines [8, 51–62].

In humans, ET raises blood pressure [63, 64] and induces vascular and myocardial hypertrophy [65–67], both risk factors for cardiovascular morbidity and mortality [68–70].

Endothelial dysfunction

The term endothelial dysfunction is widely used to describe any form of abnormal activity of the endothelium. An im-

balance of the above-mentioned vasoactive substances due to endothelium dysfunction affects vascular function negatively. Most commonly, endothelium dysfunction is characterised by an impaired NO bioavailability due to reduced production of NO by NOS or increased breakdown by reactive oxygen species [71]. In the early stages, endothelial function may be partly maintained by compensatory upregulation of prostacyclin and/or EDHF.

Endothelial dysfunction has been documented in almost every condition associated with atherosclerosis and cardiovascular disease. In humans, endothelial dysfunction has been observed in patients with hypertension [72, 73], in normotensive subjects with a family history of hypertension [74], in smokers [75, 76] and passive smokers [77], in dyslipidaemia [78, 79], in aging [73], diabetes mellitus [80–84], in obesity [84] in hyperhomocysteinemia [85, 86] and in patients with inflammatory or infectious diseases [87–89]. Many of these conditions also are characterised by overproduction of reactive oxygen species (ROS) and in turn increased oxidative stress [90]. ROS might interact with NO and reduce its bioavailability, and might directly damage cellular structures via the production of peroxynitrate. Hence oxidative stress is probably one of the major mechanisms in the development of endothelial dysfunction, if not its major contributor.

However, other factors also contribute to endothelial dysfunction, e.g. local factors such as chronic increases in shear stress, pressure and pulsatility as well as genetic predispositions and other so far unknown factors. Endothelial function therefore represents an integrated index of both the overall cardiovascular risk factor burden and the sum of all vasculoprotective factors in a given individual [91].

Given its role in the atherosclerotic process, it is not surprising that many studies demonstrate a prognostic role for endothelial function measurements in the coronary as well as in the peripheral and central circulation. First evidence came from patients with non-obstructive coronary artery disease, where two independent groups demonstrated a higher incidence of cardiovascular [92, 93] and cerebrovascular events in those with impaired coronary vascular function [94]. Coronary endothelial dysfunction predicts the incidence of further cardiovascular events even in patients without coronary artery disease [95, 96] and in heart transplant recipients [97]. Later on several other studies demonstrated incremental prognostic impact of peripheral endothelial dysfunction in patients with risk factors for coronary artery disease. Flow-mediated vasodilation was predictive for cardiovascular events beyond traditional risk factors in a large cohort of elderly patients [98], in patients with peripheral vascular disease [99], after elective vascular surgery [100], in postmenopausal women [101], in patients with chest pain [102], or in patients with coronary artery disease [103]. Similarly, venous occlusion plethysmography predicted CV events in patients with coronary artery disease [104] and in patients after acute coronary syndromes [105]. A recent study in patients with risk factors demonstrated that non-invasive peripheral arterial tonometry is able to predict late cardiovascular events [106]. However, whether peripheral endothelial dysfunction adds incremental information beyond classical risk factors in healthy humans is still debated. In a recent study in 3500 healthy subjects FMD was unable to predict the incidence of hypertension [107], whereas another study demonstrated the predictive value for incident CVD with FMD in 3026 healthy people [108].

Importantly, however, therapeutic interventions, which positively influence the cardiovascular risk profile of individuals, typically impact beneficially on endothelial function. In hypertension, for example, most classes of antihypertensive drugs improve endothelium-dependent vasodilatation in animals [109–116]. Depending on the antihypertensive drug and its pharmacological profile, improvements in endothelium-dependent vasodilatation can also be achieved in humans [117–136]. Indeed, calcium antagonists, ACE inhibitors and angiotensin-receptor antagonists, but not beta-blockers (with the exception of the NO-containing molecule nebivolol) improve endothelial function in hypertensives. Similar interventions with drugs or lifestyle changes have been studied with other risk factors and have shown similar results. The perhaps nowadays most important drugs in the prevention and treatment of atherosclerosis, the statins, have consistently been shown to improve endothelial dysfunction not only due to their lipid lowering properties but also due to their pleiotropic effects [137, 138]. Furthermore, direct infusion of reconstituted HDL is able to improve endothelial function significantly in hyperlipidemics [79].

Interestingly, not only drugs improve endothelial function in patients with cardiovascular risk factors, but also lifestyle modifications such as regular exercise [139, 140] or dietary interventions with foods rich in polyphenols, especially fruit, tea and cocoa [141–143].

Methods for assessing human endothelial dysfunction in vivo

Different techniques for measurement of endothelial dysfunction have been developed recently. The first demonstration of endothelial dysfunction in atherosclerotic coronary arteries using intracoronary infusion of acetylcholine was published in 1986 [144]. However, soon afterwards other less- and also non-invasive techniques have been developed to assess endothelial dysfunction mainly in the forearm circulation [145]. All the different techniques have

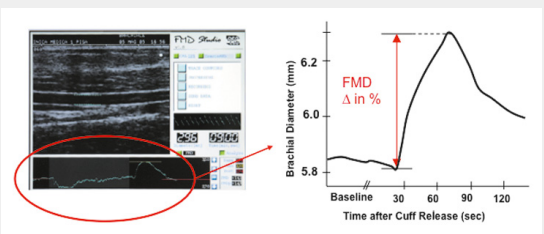

Figure 2

Flow-mediated vasodilatation.
Example of a typical ultrasound image of the brachial artery is demonstrated. Arterial diameter is shown schematically at baseline and after reactive hyperaemia induced flow-mediated vasodilatation. Blood pressure cuffs can be placed on the upper or the lower side of the transducer in the antecubital fossa, although the latter is the preferred method. On the right hand side, the time course of an FMD measurement is shown. See text for further explanation.

their advantages and disadvantages and, importantly, different vascular beds are examined. The principle, however, is simple: large conduit arteries such as the brachial or radial artery dilate in response to reactive hyperaemia (flow-mediated vasodilatation) or upon intraarterial infusion of substances such as acetylcholine (Ach), bradykinin or serotonin in the presence of a functionally intact endothelium, capable of releasing NO or other vasodilator substances (see above).

Flow-mediated vasodilatation as measured by ultrasound of the brachial artery

Due to its non-invasive properties flow-mediated vasodilatation of the forearm arteries has become the most important mode of measuring endothelial dysfunction. Many groups take advantage of this technique, which relies on the fact that endothelial cells release NO and other endothelium-derived relaxing factors in response to reactive hyperaemia (after a short occlusion of the brachial artery with a blood pressure cuff). Celermajer and his colleagues were the first to measure this response in vivo, and developed an elegant non-invasive technique to measure flow-mediated vasodilatation (FMD) of the conduit brachial or radial artery (fig. 2) [146]. We then demonstrated that indeed this response was nitric oxide dependent [20, 21] (fig. 3). The change in brachial or radial artery diameter in response to the increased shear stress induced by reactive hyperemia is measured by ultrasound technique. A feature of note is that peripheral endothelial function as assessed by FMD correlates with coronary artery endothelial function [147]. However, although the principle of this technique is simple, it is technically very challenging and therefore requires extensive training and standardization. Several attempts have been made to standardize the different protocols [148, 149].

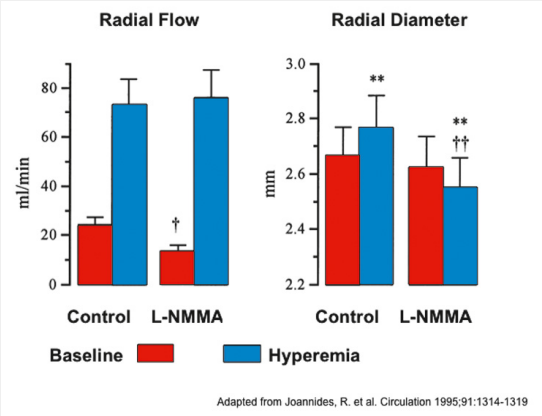

Figure 3

NO in flow-mediated vasodilatation.
Radial artery flow (mL/min) and radial artery diameter (mm) measured at baseline and during reactive hyperaemia before and after infusion of NG-monomethyl-L-arginine (L-NMMA). **P <.01 vs Base; P <.05 and P <.01 vs corresponding control value. Modified from Johannides et al. [20].

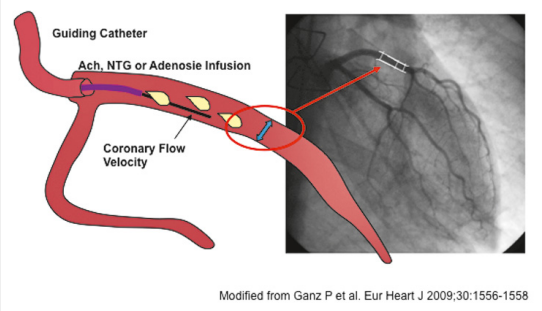

Figure 4

Measurement of coronary vascular reactivity.
Small catheters are positioned in proximal coronary arteries. Acetylcholine or nitroglycerin are infused to test conduit vessel endothelium-dependent and -independent vasodilatation respectively. Changes in vascular diameter are measured by quantitative coronary angiography. Doppler flow-velocity measurements are used to assess small vessel vasoreactivity to acetylcholine and adenosine respectively. Modified from Ganz [161].

Venous occlusion plethysmography

Although this technique is limited by its semi-invasive nature, requiring cannulation of the brachial artery, forearm venous plethysmography has the advantage that molecules, hormones or drugs can be infused intra-arterially, for instance acetylcholine (Ach) or nitroglycerine, to quantify endothelial-dependent and endothelial-independent vasodilatation respectively [72, 150]. It is of course also possible to administer other agonist and antagonists and even novel substances in a very low, systemically not effective dose into the brachial artery, with the contralateral limb serving as an internal control. Changes in forearm blood flow are measured by plethysmography in both forearms and results are expressed as the ratio of the changes in flow measured in both arms. Although the microcirculation in the forearm (which is assessed essentially by this technique) is not a target organ for atherosclerosis, it seems that the response to Ach nevertheless has an independent predictive value for future cardiovascular events [149].

Coronary endothelial function measurements

Coronary endothelial function can be measured in the catheterisation laboratory, but it is always limited by its invasive nature. Nevertheless, if applied appropriately, it provides very valuable information on the coronary vascular bed itself. Most protocols work with an intracoronary infusion of Ach to measure endothelial-dependent and nitroglycerine to measure endothelial-independent function respectively (fig. 4). As expected, coronary arteries with an intact endothelium will respond to intra-coronary Ach infusion with epicardial and microvascular dilatation, resulting in an increase in coronary blood flow. However, if the endothelial layer is dysfunctional or even interrupted, Ach produces a paradoxical vasoconstriction and a decrease in 144]. Similar to the other techniques, the response to intracoronary Ach gives important prognostic information [92].

Finger plethysmography

Because all present accepted methods of measuring endothelial function were either invasive or suffered from high inter- and intraobserver variability, other techniques in assessing vascular reactivity have been under investigation. Recently, a finger plethysmographic device which detects

pulsatile arterial volume changes has been introduced [151, 152]. A decrease in arterial blood volume in the fingertip causes a decrease in pulsatile arterial column changes, thus decreasing the measured signal and vice versa. Similarly to the assessment of endothelial function via the FMD technique by ultrasound of the brachial artery, a pressure cuff is placed on one upper arm while the other arm serves as a control. After measuring baseline blood volume changes, the blood pressure cuff is inflated above systolic pressure and is deflated after 5 minutes to induce reactive hyperaemia on one arm. The calculated index between the arm with reactive hyperaemia and the control represents a measure for endothelial function. Similar volume changes after nitroglycerin can be measured. However, augmentation of the pulse amplitude after reactive hyperaemia is a complex response to ischaemia. It may reflect changes in flow, as well as in digital microvessel dilatation, and is only partly dependent on nitric oxide [153]. Studies demonstrated that impairment of peripheral finger endothelial function is correlated with coronary microvascular function in patients with early atherosclerosis [154]. In a cross-sectional study in 1957 patients in the Framingham cohort, digital vascular dysfunction was associated with traditional and metabolic cardiovascular risk factors [155].

Pulse wave analysis

The principle of this non-invasive technique is measurement of the pulse wave and velocity profile of the propagation of the arterial wave form and its reflected wave. The central aortic wave form can be calculated as the augmentation index [156]. Although not the only contributor, endothelial function plays an important role in arterial stiffness and thus affects the results of this methodology as well. It therefore has been used to determine effects of endothelial mediators on arterial stiffness (for review see [157]). Importantly, aortic stiffness expressed as aortic pulse wave velocity is a strong predictor for future cardiovascular events and mortality, especially in those patients with a higher baseline risk, as demonstrated recently in a meta-analysis in over 15 000 subjects [158].

Other methods of assessing endothelial function

There are other imaging tools capable of assessing vascular function, including magnetic resonance technique, but these will not be described in greater detail as their importance has still to be determined.

Other possible ways of evaluating endothelial function include direct measurement of biochemical and circulating endothelium markers. One possibility is to measure the plasma levels of endothelium-derived substances directly involved in vasoconstriction and vasorelaxation (e.g. endothelin, endothelial-derived NO compounds or prostacyclin metabolites). Because atherosclerosis is believed to be, at least in part, a chronic inflammatory disease and the expression of pro-inflammatory cytokines and adhesion molecules may play an important role in the development of endothelial dysfunction, serum levels of (pro-)inflammatory markers such as C-reactive protein, interleukins and other cytokines, phospholipases and others have been measured. Due to their pro-atherogenic properties and their involvement in endothelial dysfunction, markers of oxidative stress (e.g. isoprostanes, oxLDL) or pro-angiogenic factors (e.g. VEGF) may provide further insights into early vascular changes. These markers certainly hold the potential to provide mechanistic insights, though significant confounding with other diseases and conditions has to be taken into account.

Recently circulating endothelial progenitor cells (EPC) have emerged as a powerful marker of endothelial dysfunction. Indeed, endothelial dysfunction may reflect an imbalance between vascular injury and EPC-based repair [5, 159] and might indicate pre-clinical endothelial damage and a target for vascular protection [149]. Furthermore, genetics may provide more insights into the molecular mechanisms of endothelial function. In particular, single nucleotide polymorphisms of endothelial genes such as endothelin-1, eNOS among others, have been studied [149]. In the future, gene polymorphisms might help to further refine individual risk assessment for endothelial dysfunction and future events. For instance, coronary endothelial dysfunction in patients with coronary artery disease correlates with cytochrome P450 polymorphisms [160].

The future of endothelial dysfunction

In the last 30 years, an enormous number of studies have been published on endothelium function in experimental animals and humans. Currently, a search in PubMed results in 175 454 displayed studies, more than 10 000 in the year 2009 alone. This underscores the persistent interest and importance of endothelial research. Future areas of interest are the relation of endothelial dysfunction to endothelial progenitor cell number and function and their therapeutic modulation, as well as genetic factors influencing endothelial function. Finally, the assessment of endothelial function remains an important tool for assessment of the vascular effects of novel therapeutic agents in their clinical development.

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