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Evolving Pathophysiological Perspectives in Endothelial Dysfunction

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Cardiovascular disease is responsible for the majority of morbidity and mortality in the Western world. Most forms of cardiovascular disease involve atherosclerotic vascular changes in the coronary, cerebral, renal and peripheral circulation. Hence, the understanding of vascular dysfunction and its role in the development of target organ damage is of great clinical interest.

Furchgott and Zawadzki's[1] seminal discovery 20 years ago that the endothelium plays an obligatory role in vascular relaxation not only revolutionised cardiovascular physiology, but also stimulated an evolving understanding of the development of cardiovascular disease. Soon after the first studies showing the importance of the endothelium were published, substantial evidence demonstrated that the endothelium-dependent responses were impaired in animal models and in patients with cardiovascular disease. The working hypothesis derived from these observations is that endothelial cell dysfunction plays a key role in the initiation and progression of cardiovascular disease. Studies that address this hypothesis continue to provide further insight into the pathophysiological mechanisms and to suggest new therapeutic strategies.

Endothelial cells actively regulate basal vascular tone in physiological and pathological conditions by responding to mechanical forces and neurohumoral mediators with the release of a variety of relaxing and contracting factors.[2] The endothelium-derived relaxing factors (EDRFs) include nitric oxide (NO), prostacyclin and an, as yet elusive, endothelium-derived hyperpolarising factor. These substances are also able to inhibit platelet function as well as proliferation of smooth muscle cells. On the other hand, endothelial cells may produce vasoconstrictors and growth promoters such as angiotensin II, endothelin-1 (ET-1), and prostaglandin H_2 (PGH_2). Thus, the activity of the endothelium extends far beyond the control of vascular tone and reactivity, and the release of vasodilating mediators clearly reflects only one aspect of the homeostatic and protective role of the endothelium. Nevertheless, endothelium-dependent, NO-mediated vasodilation is generally used as a reproducible and accessible parameter to probe endothelial function in different pathophysiological conditions.

NO is the principal factor involved in the antiatherosclerotic properties of the endothelium.[3] NO interferes *in vitro* with key events in the development of atherosclerosis, such as monocyte and leucocyte adhesion to the endothelium, as well as platelet-vessel wall interaction.[4-6] Finally, NO has been shown to inhibit vascular smooth muscle cell proliferation and migration *in vitro* as well as *in vivo*.[7,8] In agreement with these findings, inhibition of the NO-producing enzyme, nitric oxide synthase (NOS), caused accelerated atherosclerosis in experimental models.[9] Major risk factors for atherosclerotic vascular disease, such as diabetes mellitus, hypertension and hypercholesterolaemia, have been associated with impaired NO availability.[10] There is indeed evidence that in the presence of cardiovascular risk factors, the protective role of the endothelium is diminished, whereas the production of vasoconstrictive, proaggregatory and promitogenic mediators is maintained or enhanced.

1. Nitric oxide (NO) Availability and Hyperglycaemia

The relationship between diabetes and premature cardiovascular disease is well established.[11] Atherosclerosis occurs earlier in diabetics than in non-diabetics and is more severe and diffuse in the former group.[12] Diabetic microvascular disease contributes to common complications such as retinopathy and nephropathy. Although the link between hyperglycaemia and cardiovascular disease is not understood, loss of the modulatory role of the endothelium may be implicated in the pathogenesis of diabetic vascular disease.

Hyperglycaemia is clearly recognised as the initiating insult in the pathogenesis of diabetic complications. Several studies have shown impairment of endothelium-dependent relaxations in response to various receptor-mediated vasodilators in different vas-

cular beds from diabetic animals.[13,14] Abnormal endothelial cell function appears to be associated specifically with hyperglycaemia rather than with any other potential metabolic disturbance. Indeed, substantive evidence linking hyperglycaemia and endothelial cell dysfunction comes from *in vitro* incubation studies in which exposure of arteries to elevated concentrations of glucose caused endothelial dysfunction similar to that observed in diabetic animals.[15,16]

Although high concentrations of glucose may exert hyperosmolar effects, the impaired endothelial response is not due to hyperosmolarity, because similar concentrations of mannitol have no effect on endothelium-dependent relaxations.[15] Hyperglycaemia-induced endothelial dysfunction may result from decreased production of endothelium-derived NO, inactivation of NO by oxygen-derived free radicals and/or increased production of endothelium-derived contracting factors (EDCFs), which oppose the vascular effect of NO. The findings that endothelium-dependent relaxations were restored in the presence of cyclo-oxygenase inhibitors, such as indomethacin or PGH_2/thromboxane A_2 (TXA_2) receptor antagonists, suggest that the synthesis and/or release of NO is not altered in diabetes.[15,16] It is also unlikely that these impaired endothelium-dependent relaxations are due to decreased responsiveness of smooth muscle cells, because relaxations caused by direct smooth muscle vasodilators are not affected in the course of experimental diabetes.[13-15]

The reasons for decreased bioavailability of endothelium-derived NO are still unknown but may involve breakdown of NO by reactive oxygen species (ROS). Increased production of ROS and decreased free radical scavenger systems have been described in diabetes. That free radicals participate in the deleterious effects of elevated glucose on the endothelium has been suggested by several studies showing that a number of free radical scavengers prevent and restore impaired endothelium-dependent relaxations in diabetic animals as well as in normal arteries exposed to elevated glucose.[17,18] Despite uncertainty regarding the precise mechanisms of generation and release of free radicals in diabetes, it is clear from these animal models that oxidative stress is an important contributor to the development of diabetic vascular endothelial cell dysfunction. Similar impairment of endothelium-dependent vasodilation has been demonstrated in patients with type 1 diabetes in the absence of clinical complications.[19] Blunted relaxation of the corporal smooth muscle of the penis in response to acetylcholine was found in diabetic men with impotence.[20] Acetylcholine-induced vasodilation was reduced in patients with type 2 diabetes, implying abnormalities in the NO pathway.[21]

2. NO Availability and High Blood Pressure

Hypertension is associated with an increase in pressure on the arterial side of the circulation system, mostly caused by elevated peripheral resistance determined by the contractile state of the resistance arteries with a diameter of ≤200μm. The resistance arteries are affected by neuronal stimulation, circulating hormones and paracrine/autocrine mechanisms within the blood vessel wall. Normally, the vasculature is in a constant state of vasodilation due to the basal formation of NO by endothelial cells.[22] However, the endothelium might directly increase peripheral resistance via an enhanced release of constricting factors or a reduced production or enhanced breakdown of NO. In 1990, two independent groups showed that endothelium-dependent vasodilation, assessed by venous occlusion plethysmography, was impaired in humans with essential hypertension.[23,24] The response to acetylcholine, but not to sodium nitroprusside, was attenuated in the forearm vascular bed of hypertensive patients. Later, this finding was confirmed in the coronary circulation.[25,26] Impaired vasodilation has also been found using the brachial artery high-resolution ultrasound technique during reactive hyperaemia.[27] Impaired endothelium-dependent vasodilation has been found in resistance or conduit arteries in a total of more than 20 studies in human hypertension. Accordingly, plasma levels of NO are reduced in patients with essential hypertension.[28] In only two studies, was the impaired endothelium-dependent vasodilation in hypertensive patients not confirmed.[29,30] Similar reductions were also seen in patients with primary aldosteronism and renovascular hypertension.[31] The response to sodium nitroprusside has, with a few exceptions, been reported to be preserved, suggesting that no major impairment in endothelium-independent vasodilation exists in human hypertension.

A decline in endothelium-dependent vasodilation with age, similar to that observed in healthy individuals, has been reported in hypertensive patients, but at any age vasodilation was lower in the hypertensive individuals,[32] suggesting that high blood pressure accelerates the effect of aging on the endothelium. In patients with essential hypertension, the impaired response to acetylcholine in the forearm circulation can be improved by indomethacin, indicating that cyclo-oxygenase-dependent vasoconstrictor prostanoids also contribute to impaired endothelium-dependent relaxation in hypertensive patients.[32,33] Moreover, besides EDCFs such as TXA_2 and PGH_2, oxygen-free radicals can play an important role in endothelial dysfunction in hypertension. Indeed, the antioxidant vitamin C has been found to improve endothelial vasodilator function in the forearm and the coronary circulation by an NO-dependent mechanism in patients with essential hypertension.[34]

It has not yet been fully clarified whether the endothelial dysfunction precedes the development of hypertension, or whether it is a consequence of high blood pressure. Young normotensive subjects with a family history of hypertension showed attenuated vasodilation when compared with controls without a family history of hypertension.[35] In contrast, the vasodilatory response was restored in patients with primary aldosteronism following successful surgery and normalisation of blood pressure.[31] Whether the endothelial dysfunction is a cause or a consequence of high blood pressure remains a controversial issue. However, it is clear that such an impairment plays a crucial role in determining the cardiovascular risk of hypertensive patients.

3. NO Availability and Hypercholesterolaemia

High levels of low-density lipoprotein (LDL)-cholesterol have invariably been linked to increased cardiovascular mortality. In 1990 it was confirmed in organ chamber experiments that lysophosphatidylcholine, a component of oxidised LDL-cholesterol, was directly responsible for impaired endothelium-derived NO activity in rabbit aortic strips. Accordingly, in humans, hypercholesterolaemia was accompanied by impaired endothelium-dependent vasodilation in both coronary and forearm vascular beds.[36-41] In hypercholesterolaemia, impaired NO activity can indicate either decreased formation or increased degradation. Lysophosphatidylcholine, which accumulates during oxidative modification of LDL, has been shown to interfere with the Gi-protein-dependent signalling pathway.[41] Since numerous agonists of NO release mediate their effect through a Gi-protein-dependent pathway,[42] this will result in decreased formation of NO. There is also evidence of reduced transcription and enhanced breakdown of NOS transcripts with increasing concentrations of oxidised LDL.[43] Finally, hypercholesterolaemia is associated with increasing circulating concentrations of asymmetric dimethylarginine, an endogenous inhibitor of NOS. This has been demonstrated in hypercholesterolaemic rabbits and humans.[44,45] This is particularly interesting because these observations suggest that administration of L-arginine may overcome a competitive inhibition of NOS. In support of this theory, administration of L-arginine increases synthesis of NO by the vascular endothelium[46] and improves NO-dependent vasodilation in conditions such as hypercholesterolaemia and angina pectoris.[40,47] Alternatively, reduced NO activity could be caused by enhanced catabolism.

4. Endothelial Dysfunction and Atherogenesis

Endothelial injury is now regarded as an important initial event in atherogenesis.[3] Besides the above-mentioned risk factors, other conditions have been shown to disrupt endothelial integrity. Active cigarette smoking,[48] and even prolonged exposure to environmental tobacco smoke,[49] has been shown to be associated with impaired endothelium-dependent dilation in otherwise healthy adults. Aging, too, has been associated with progressive endothelial impairment,[50] and this age-related dysfunction appears to occur earlier in men than in women.[51] Hyperhomocystinaemia, which causes chemical endothelial injury, is associated with premature atherosclerosis and thrombosis. The finding that many of these conditions, which are associated with clinical progression of vascular disease, are clearly related to endothelial dysfunction has added weight to the 'response to injury' theory by Ross.[3]

The consequences of endothelial damage, which promote fatty streak and plaque formation, include increased adherence of monocytes, increased permeability to monocytes/macrophages and lipoproteins, which then accumulate in the vessel wall, increased platelet adhesion and aggregation, and smooth muscle cell migration and proliferation.[3] Endothelial dysfunction is characterised by decreased bioavailability of NO. NO is synthesised from L-arginine by constitutive NO synthase (cNOS) through a five-electron oxidation of the guanidine-nitrogen terminal of L-arginine.[52] The activity of the L-arginine/NO pathway is a balance between synthesis and breakdown of NO by its reaction with superoxide anion (O_2-). Under physiological conditions, the production of this molecule is not affected by O_2-. Hence, the endothelium-derived NO may exert its well-known vascular protective effects favouring an antiatherosclerotic environment. However, in the presence of cardiovascular risk, an excessive production of O_2- occurs. O_2- rapidly inactivates NO, leading to the formation of high concentrations of peroxynitrite ($ONOO^-$), a very powerful oxidant.[10]

High concentrations of $ONOO^-$ are very toxic, as $ONOO^-$ can form peroxynitrous acid, the cleavage products of which are among the most reactive and damaging species in the biological system.[53] Taken together, these data indicate that catabolism of NO by its reaction with O_2- could be an important mechanism underlying endothelial dysfunction and oxidative vascular injury described in a number of vascular diseases.[54] It can be postulated that harmful concentrations of $ONOO^-$ can be achieved in a dysfunctional endothelium in which O_2- generation is increased by cyclo-oxygenase, xanthine oxidase, and NADH oxidoreductase.[55-57] However, recent evidence also indicates that a dysfunctional NOS may lead to a shift in the balance between the production of protective NO and deleterious oxygen-derived free radicals.[10,58]

5. Calcium Antagonists and Endothelial Function: A New Target for Cardiovascular Therapeutics

During the last 10 years, we have seen an exponential growth in our knowledge and understanding of the role of endothelial dysfunction in a variety of cardiovascular conditions. It has been demonstrated that endothelial dysfunction might indeed be the initiating event in the process of atherosclerosis and vascular remodelling, which subsequently leads to clinical coronary artery disease. Accordingly, there has been an ongoing, aggressive search for therapeutic choices suitable for reversing endothelial dysfunction in the hope that such therapeutic intervention, if instituted early in the course of the disease, might prevent and/or modify the subsequent risk of clinical disease and related cardiac events. Indeed, the time has come to recognise the endothelium as an important new target for cardiovascular therapeutics.

Under acute conditions, calcium antagonists do not affect the release of endothelium-derived vasoactive substances, although the production of these factors in endothelial cells is associated with an increase in intracellular calcium.[59] In contrast to their effects in vascular smooth muscle, calcium antagonists do not influence intracellular calcium regulation in endothelial cells. Intracellular calcium release and transmembranous calcium influx upon stimulation of endothelial cells by agonists are not affected by calcium antagonists. This is due to the fact that endothelial cells apparently do not possess voltage-operated, L-type calcium channels.[60] Therefore, calcium antagonists do not appear to have any adverse effect on the calcium-dependent formation of NO. On the contrary, during long-term administration they may improve endothelial function and structure, as observed in the aorta and the mesenteric resistance arteries of an experimental model of hypertension (L-NAME-induced hypertension).[61-63] Calcium antagonists may therefore facilitate the effects of EDRFs at the level of vascular smooth muscle, as has been suggested by enhanced sodium nitroprusside-induced relaxations in certain conditions.[61]

In addition, calcium antagonists seem to interfere with the vasoconstrictor effects of ET-1 and cyclo-oxygenase-derived contracting factors.[64] In the porcine coronary artery, ET receptors are linked to voltage-operated calcium channels via a G-protein, and calcium antagonists inhibit ET-induced vasoconstriction in this blood vessel.[65] Furthermore, infusion of nifedipine and verapamil prevents the vasoconstriction caused by ET-1 in the forearm circulation of healthy volunteers.[66]

Several investigations have demonstrated a positive effect of these compounds on endothelial dysfunction in an animal model of experimental hypertension.[61-63] Only a few studies, however, have assessed the effects of calcium antagonists on endothelial function in hypertensive patients. In patients with essential hypertension, oral treatment with lacidipine increased forearm vasodilation in response to acetylcholine and bradykinin, suggesting that this drug can improve endothelial function in patients with essential hypertension.[67] In addition, Frielingsdorf et al.[68] reported that nicardipine, a dihydropyridine, and diltiazem, a benzothiazepine, can improve the impaired endothelium-dependent vasomotor response induced by exercise in atherosclerotic stenotic coronary vessels of normotensive patients and in normal and stenotic vessels of patients with essential hypertension. Furthermore, it was demonstrated that treatment with the dihydropyridine calcium antagonist nifedipine, but not with the β-blocker atenolol, prevents endothelial dysfunction, assessed as relaxations induced by acetylcholine in resistance-size small arteries dissected from a gluteal biopsy of essential hypertensive patients.[69]

Several hypotheses can be put forward to clarify the mechanism through which calcium antagonists can improve endothelial function. Firstly, such a beneficial effect may be exerted on different agonists, indicating that the mechanism involved is not related to an interaction with surface endothelial receptors or selective intracellular signal transduction pathways. Calcium antagonists could potentially act directly on NOS, because the enzyme activity is calcium-dependent. According to this theory, however, these drugs would block and not increase NO production. This unfavourable possibility is ruled out because endothelial cells do not express voltage-operated calcium channels.[60] An alternative explanation is that they could enhance NO-induced vasodilation by decreasing calcium influx into smooth muscle cells in which L-type channels are present.[60] Finally, calcium antagonists may have effects that differ from the effect of calcium influx blockade, and these could be beneficial in reversing endothelial dysfunction. Indeed, this class of drugs has been shown to have antioxidant properties,[70-72] a mechanism through which they could protect endothelial cells against free radical injury.

A meta-analysis of studies in patients with hypertensive stroke and transient ischaemic attack indicates that antihypertensive therapy reduces stroke recurrence by 38%.[73] Calcium antagonists not only reduce systolic blood pressure and the incidence of stroke-related mortality in humans, but have been demonstrated to exert additional protective effects against stroke in stroke-prone spontaneously hypertensive rats (SHRSP),[74-76] which is a good model for mechanistic and interventional studies because the cerebral lesions in these animals are similar to those in humans. Dihydropyridine calcium antagonists vary in their chemical structure and antihypertensive effect, but contain aromatic rings that account for their antioxidant activity.[70-72] A recent study has shown that, in the SHRSP model, these drugs reduce plasma and LDL oxidation and prolong survival independently of blood pressure modifications.[77] In this regard, we recently observed that

nifedipine inhibits superoxide production induced by pulsatile stretch in human aortic endothelial cells.[78] Thus, nifedipine may affect mechanical forces that, as determinants of the balance between NO and superoxide, are likely to play a key role in the pathophysiology of hypertensive vascular disease. Such a hypothesis is confirmed by recent evidence in humans showing that nifedipine increases endothelium-dependent vasodilation in patients with essential hypertension by restoring NO availability and preventing the facilitating effect of the antioxidant vitamin C.[79] In the same study, nifedipine treatment also decreased plasma values of lipoperoxides and isoprostanes.[79] Furthermore, nifedipine improves vasodilation in response to acetylcholine in the forearm circulation of normotensive patients with hypercholesterolaemia,[80] a positive effect obtained without modifications in blood pressure values or lipid profile.

Taken together, these findings have important clinical implications. Indeed, it has been established that endothelial dysfunction is a promoter of the pathogenesis of atherosclerosis, suggesting that reversal of this condition could represent an important target for pharmacological prevention of atherosclerotic vascular disease associated with cardiovascular risk factors. In line with this possibility, both the International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT)[81] and the Montreal Heart Study[82] demonstrated a reduction in the number of new coronary lesions on angiography with calcium antagonist treatment. In addition, recent trials such as the Evaluation of Nifedipine and Cerivastatin on Recovery of Endothelial Function (ENCORE I) have demonstrated a beneficial effect of nifedipine alone on endothelium-dependent vasodilation in epicardial arteries of patients with ischaemic coronary disease,[83,84] whereas the ongoing ENCORE II is investigating, in a similar patient population, the effect of nifedipine treatment alone or in combination with a statin on the development of atherosclerotic structural lesions.[83] Although the recent Prospective Randomized Evaluation of the Vascular Effects of Norvasc Trial (PREVENT) did not show any demonstrable effect on angiographic progression of coronary atherosclerosis, amlodipine significantly reduced the rates of unstable angina and coronary revascularisation.[85] Furthermore, the positive results of the European Lacidipine Study on Atherosclerosis (ELSA),[86] which evaluated the long-term effect of treatment with lacidipine on carotid morphology in hypertensive patients provide additional support for the beneficial effect of calcium antagonists on the vessel wall.

6. Future Directions

Undoubtedly, further research in this area is required. First, we need to have more studies that carefully examine the relationship between the vascular response to various stimuli in the peripheral circulation and the response observed in the coronary circulation in the same individuals. Before any significant clinical emphasis is given to the beneficial effects of various cardiovascular therapies on endothelial function, it is essential to demonstrate that the presence of endothelial dysfunction is predictive of subsequent risk of cardiovascular events. We are starting to get this evidence.[85-87] However, we still need randomised clinical trials to critically examine the effects on clinical outcome of drugs that improve endothelial function. The rapidly emerging interest regarding the vascular protective effects of different therapeutic strategies, including calcium antagonists, makes it crucial for such trials to be conducted soon, so that clinicians can prescribe the appropriate therapy on the basis of clinical evidence.

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