RATIONALE FOR PROMISING NOVEL THERAPEUTIC APPROACH FOCUSED ON ENDOTHELIN PATHWAY FOR PERIPHERAL ARTERIAL DISEASE

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ABSTRACT

There is evidence to suggest that endothelin (ET-1) is involved in the pathophysiology of peripheral arterial disease (PAD), contributing to atherosclerotic narrowing of the lower limb arteries as well as microvascular dysfunction. This paper summarises the evidence and discusses the potential role of a promising novel therapeutic strategy for PAD focused on ET-1 pathway modification. ET-1 pathway is involved in PAD with raised plasma levels and local sources of ET-1. More recent evidence of a potential role of ET-1 in ischaemia-induced skeletal muscle damage suggests that this may be a useful target for treatment. ET antagonism may play an adjunctive role in improving endothelial function and reducing oxidative tissue damage within the affected vessels. However, in patients with advanced atherosclerotic lesions, manipulation of the ET-1 pathway is unlikely to be of a significant benefit in terms of lesion regression and improving blood flow. Results from small clinical studies support data from promising initial pilot and basic research. Considering the potentially important role of ET-1 in the development of vascular dysfunction reviewed in the present article – conditions with increased inflammatory activity, oxidative stress, and vascular tone such as atherosclerosis, and PAD – larger clinical trials using ET receptor antagonists are encouraged and needed.

Keywords: Peripheral arterial disease, endothelin, cause-based treatment.

INTRODUCTION

Peripheral arterial disease (PAD) is defined as the set of vascular diseases caused primarily by atherosclerosis (AS) and thromboembolic pathophysiological processes that alter the normal structure and function of the aorta, its visceral branches, and arteries of the lower limbs.[1] In Western society, >20% of the population over the age of 55 are affected by this condition.[2] One-third of patients with PAD have intermittent claudication (IC),[3] defined as leg pain during exercise caused by arterial occlusive disease, which leads the patient to stop, and decreases with rest. In severe PAD, critical limb ischaemia (CLI) occurs, where viability of the limb is threatened, and 20% of patients face limb loss within a year.[4] There is evidence to suggest that endothelin (ET-1) is involved in the pathophysiology of PAD, contributing to atherosclerotic narrowing of the lower limb arteries as well as microvascular dysfunction. This paper summarises this evidence and discusses the potential role of a promising novel therapeutic strategy for PAD focused on ET-1 pathway modification.

ET-1 Pathway

ET-1 production and secretion are primarily controlled at the gene transcriptional level (Figure 1). ET-1 gene expression is regulated by a number of transcription factors, including activator protein 1, hypoxia inducible factor-1, nuclear factor κB (NF-κB), vascular endothelial zinc finger 1, GATA binding protein 2 (GATA-2), and GATA-4, nuclear factor of activated T cells among others that are of relevance for AS and diabetes. The transcription factors are, in turn, activated by several inducers such as angiotensin II, cytokines, glucose, insulin,

and hypoxia. Mature ET-1 is formed from pre-pro-ET-1 via a 39-amino acid intermediate, big ET-1.[5] Big ET-1 is processed to ET-1 by a family of ET converting enzymes (ECEs) and other enzymes such as chymases, non-ECE metalloproteinases, and endopeptidases.[5,6] Under physiological conditions, ET-1 is produced in small amounts mainly in endothelial cells, primarily acting as an autocrine and/or paracrine mediator. Under pathophysiological conditions, however, the production is stimulated in several cell types such as endothelial cells, vascular smooth muscle (VSM) cells, cardiac myocytes,[7] and inflammatory cells.[8,9] Increased expression of ET-1 has been demonstrated in AS animal models[10,11] as well as in human coronary artery disease (CAD)[12,13] and PAD.[14] This results in enhanced vasoconstrictor tone, increased inflammatory activity, and elevated oxidative stress. The effect of ET-1 is mediated via activation of its two distinct receptors, the ET Type A and B (ETA and ETB) receptors. In the vascular wall the ETA receptor is localised to the smooth muscle cell and mediates the major part of the vasoconstrictor effect of ET-1 under physiological conditions. The ETB receptor is localised to the endothelial cells and mediates vasodilatation via release of nitric oxide (NO). ETB receptors are also located on VSM cells and mediate vasoconstriction.

ENDOTHELIAL DYSFUNCTION AND INFLAMMATION AT THE PATHOPHYSIOLOGY OF PAD

Endothelial dysfunction is considered to occur early during the development of cardiovascular disease (CVD) including AS and vascular complications associated with diabetes mellitus. A key event in endothelial dysfunction is the reduction in bioavailability and biological activity of NO. Reduced levels of NO contribute to increased vascular tone, inflammation, platelet aggregation, and oxidative stress, which all are central features of AS and diabetic vasculopathies.[15] Development of endothelial dysfunction involves several biological mediators including increased expression of ET-1 and altered expression of ET receptors.[16] Considering the prominent biological actions mediated by ET-1, such as potent vasoconstriction, pro-inflammatory actions, and mitogenic properties, overproduction of ET-1 may be of significant pathological importance in CVD (Figure 2).

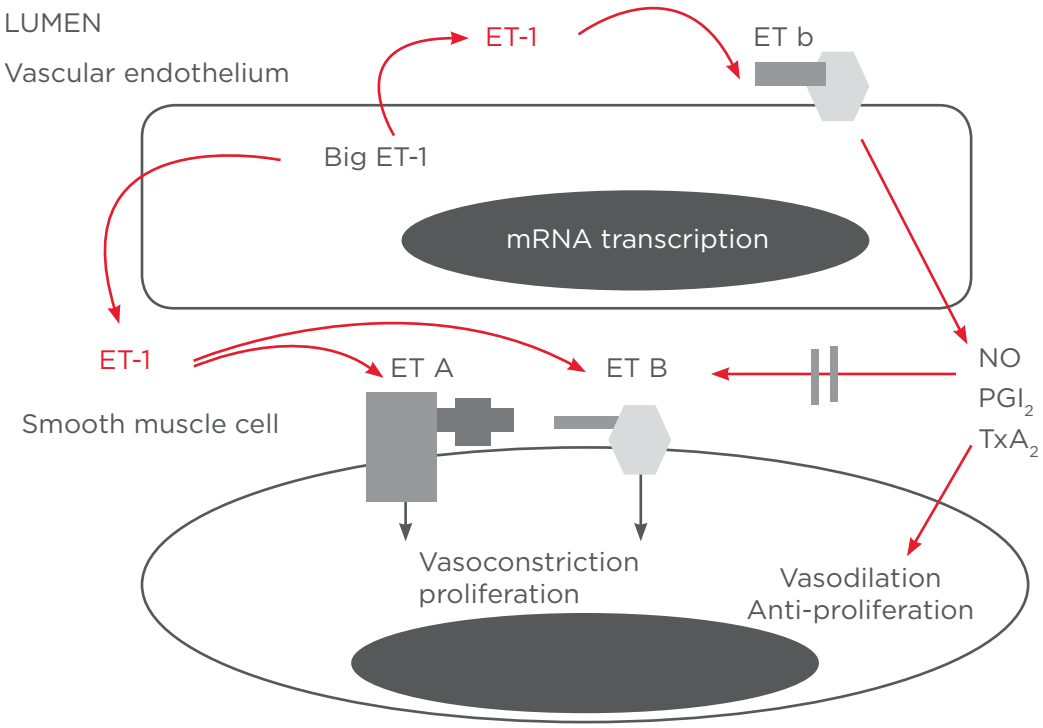

Figure 1: Endothelin pathway in the vessel wall.
ET-1: endothelin; ET A/B: endothelin receptor type A/B; NO: nitric oxide; PGI_2: prostacyclin; TxA_2; thromboxane A2.

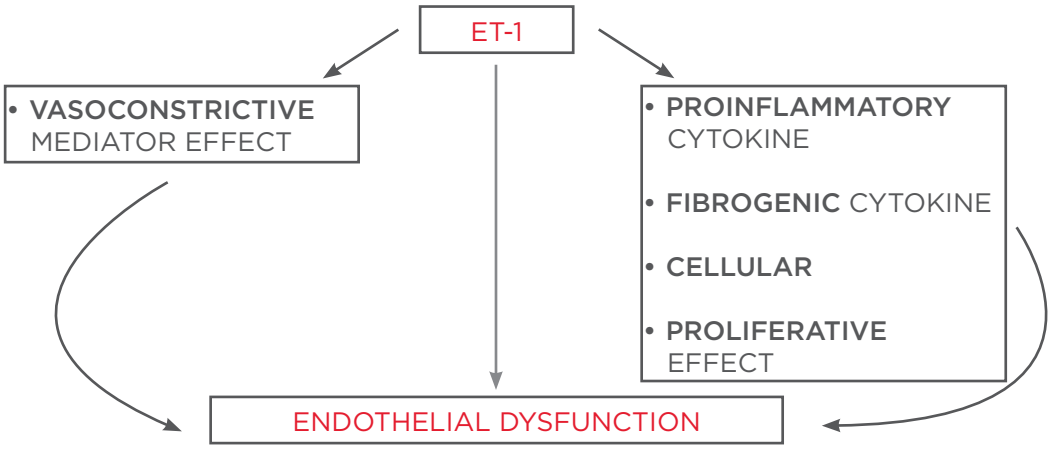

Figure 2: Endothelin (ET-1) biological actions.

The endothelium seems to be responsible for the balanced relationships involved in the functioning of the vascular wall. When this balance is upset, the regulation of vascular homeostasis is lost, causing endothelial dysfunction, defined as functional deterioration of the endothelium characterised by vasospasm, vasoconstriction, abnormal coagulation mechanisms, abnormal fibrinolysis, and an increase in vascular cell proliferation. A reduction in the brachial arterial flow mediated dilation (BAFMD), a surrogate of endothelium function, has been demonstrated in patients with PAD[17] with no significant differences according to the severity of the disease,[18,19] supporting the hypothesis that endothelial dysfunction is a process which begins in the early stages of PAD. Moreover, elevated high-sensitivity C-reactive protein (hsCRP) seems to be an independent predictor of cardiovascular (CV) events in patients with PAD.[17]

The endothelium homeostasis is, in part, mediated by NO levels. There is an inducible isoform of nitric oxide synthase (iNOS), which is stimulated by cytokines and produces much larger quantities of NO than the other isoforms.[20] In order to work, these enzymes require cofactors, including tetrahydrobiopterin (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH). The importance of NO in atherogenesis was suggested after studies in mice with an apolipoprotein E (apoE) deficiency found that the atherosclerotic lesions developed spontaneously when endothelial nitric oxide synthase (eNOS) was eliminated. However, in studies with apoE and iNOS double-knockout mice, there was a reduction in the formation of the atherosclerotic plaque. These findings suggest that eNOS-derived NO may 'protect' the vascular wall from AS, while the NO deriving from iNOS may promote the formation of atherosclerotic lesions.[20,21]

Once released into the lumen of the vessel, the NO deriving from the endothelium is oxidised or participates in nitrosylation reactions. The NO activity is the result of the balance between its production by NOS and its inactivation by oxygen free radicals. We know that ET-1 and its receptors are involved in the expression of such NOS and in the NO levels. Therefore, ETA inhibits NO synthesis, whereas ETB increases NO production and release (Figure 1).[22] However, there is a substantial change in the expression and function of the ET receptors in various pathophysiological conditions as AS, resulting in an altered biological response.[16]

Oxidised low-density lipoprotein (LDL) also increases the production of ET-1.[23] High levels of ET-1 have been found in patients with arteriosclerosis, both in coronary disease[24,25] and PAD.[26] In a previous study, as occurred with BAFMD, we found no relationship between the nitrite levels in plasma, as estimator of NO production, and the severity of the PAD.[27] This leads us to think that the loss of the physiological function of NO as a homeostatic signal by which the endothelium acts, occurs in the first stages of PAD and that this is perpetuated through a vicious circle (self-feedback), which leads to a reduction in BAFMD as estimator of endothelial dysfunction. Otherwise, we have seen that elevated hsCRP has a linear association to the clinical severity with which PAD presents.[28] We not only found this linear

association between clinical severity and elevated hsCRP, but we also found that, although weak, there seems to be a reverse correlation between hsCRP levels and BAFMD values in these patients.

Therefore, the CRP stimulates the production of NO by NOS, increasing NO oxidation and nitrosylation and reducing the levels of BH4, encouraging the formation of free radicals. These free radicals, in turn, inactivate the NO which has been produced and destroy the BH4, resulting in endothelial dysfunction. In fact, it has been demonstrated in *in vitro* studies, that CRP is capable of stimulating the production of NO, independent of iNOS stimulation.[29] We observed increased levels of hsCRP in the patients with PAD and also a linear correlation between these levels and the clinical severity degree.[27] This finding suggests the existence of an inflammatory substrate in the aetiopathogenesis of PAD. Both CRP, as principal indicator of systemic inflammation, and other cytokines (interleukin [IL]-6, tumour necrosis factor [TNF-α] etc.), may be responsible for the loss of balance in the endothelial NO system and the subsequent endothelial dysfunction. The reverse correlation between BAFMD and the hsCRP levels found in this study, although weak, implies a relationship between inflammation and endothelial dysfunction in the aetiopathogenesis of PAD, with the loss of the homeostatic function of NO as a key step in the origins of the disease, but not in its progression.

Summarising, when BAFMD is used as a surrogate measure of endothelial dysfunction, our observations suggest that endothelial function during the initial stages of PAD may not progressively deteriorate with disease severity.[19] Concentrations of the inflammatory marker CRP, however, exhibited a great correlation with PAD progression. These observations support the previously documented role of inflammation in the maintenance and progression of PAD.[28]

These findings highlight the potential importance of early intervention during the initial stages of PAD in order to delay disease progression. As a prototypical target for therapeutic intervention, patients with early PAD may benefit from reduced concentrations of ET-1. Concentrations of ET-1 can be decreased using renin-angiotensin inhibitors, which indirectly block ET-1 production, and statins, which reduce ET gene expression irrespective of their lipid-lowering effects.[30,31] Treatment with dual and single ET receptor antagonists (i.e. bosentan, zibotentan) may also confer benefits in the future and merit evaluation, either alone or in combination with other drugs that delay PAD progression.

POTENTIAL ROLE OF ET-1 IN PAD

In PAD, raised ET-1 levels have been demonstrated in both patients with claudication and CLI.[32] Mangiafico et al.[32,33] found that whilst no correlation between plasma ET-1 levels and pain-free walking distance in patients with claudication was found, treating claudicants with prostaglandin E1 resulted in improved walking distances, which was associated with decreases in ET-1 plasma levels. Recently, our group confirmed that raised ET-1 plasma levels occurred in patients with PAD compared to controls. Our study found higher ET-1 plasma levels in patients with claudication compared to those with CLI.[34] This suggests that in very advanced disease, as vessel damage progresses, potential sources of ET-1 such as endothelial cells may be lost, leading to reduced ET-1 secretion. Newton et al.[35] studied markers of endothelial function in patients with CLI before and after lower limb amputation and found that ET-1 plasma levels, unlike those of vascular endothelial growth factor and von Willebrand factor, did not reduce following amputation.[10] Moreover, higher levels of ET-1 in these patients were associated with poorer prognosis in terms of all-cause mortality and CV mortality.

ET-1 immunoreactivity, mRNA levels,[36] and ET receptor binding[37] have been found in atherosclerotic plaques and diseased coronary arteries where ET-1 was associated with medial VSM cells and luminal endothelial cells. Indeed, VSM cells could be a potential source of ET-1 in AS, which would explain that the development of the disease is associated with increased production of ET-1 even if there is loss of ET-1 production by endothelial cells, except in the advanced stages of the disease. Meanwhile, ETA receptors were found predominantly on smooth muscle cells and ETB receptors on microvascular endothelial cells.[38]

In diseased femoral and popliteal arteries obtained from patients with CLI, a similar pattern has been shown with ET-1 binding to ETA and ETB receptors on medial VSM cells and further ETB receptors located on microvessels and vascular nerves.[39] These atherosclerotic lesions are significant sources of ET-1. Whilst ET-1 is known to be a paracrine

factor, being released abluminally to act on ET receptor bearing cells locally, overspill of the peptide into the systemic circulation are likely to contribute to raised plasma levels and enables the peptide to also exert its effect further downstream. The effects of ET-1 on vessels and blood flow are well established. Infusion of ET-1 into femoral arteries in dogs resulted in an initial increase followed by a gradual decrease in femoral blood flow.[40,41] In humans, ET-1 infusion reduced blood flow in the legs of young healthy volunteers.[42] In older subjects, ET antagonism resulted in greater increases in blood flow than in younger subjects, suggesting that ET-1 may play a role in the age-related raised baseline vascular tone.[43] However there is currently little evidence on the direct effect of ET-1 on femoral blood flow in patients with PAD.

PAD is characterised by endothelial dysfunction and AS in the lower limb arteries,[44] and ET-1 is likely to contribute to both of these processes. ET-1 activation is associated with atherogenic risk factors such as hypertension, hyperlipidaemia,[45] and diabetes[46] where, as a potent vasoconstrictor, it acts to antagonise endothelium-derived vasodilators such as nitric oxide contributing to endothelial dysfunction.[47-49] Endothelial dysfunction in turn, promotes leucocyte adhesion, thrombosis, inflammation, and cell proliferation leading to the development of atherosclerotic plaques. Once developed, these lesions provide further sources of ET-1 which acts, in a paracrine fashion, to contribute to the progression of the disease.[50] As the atherosclerotic lesions progress, flow-limiting stenoses or even occlusions occur, compromising perfusion to the distal tissue.

INCREASED OXIDATIVE STRESS BY ET-1

Several reports support a role for ET-1 in the formation of reactive oxygen species (ROS). Formation of superoxide (O_2-) will result in decreased bioactivity of NO and formation of peroxynitrite. ET-1 stimulates ROS production in human endothelial and VSM cell cultures,[51,52] as well as in isolated vessels.[53-55] Mainly ETA receptors seem to mediate ROS production stimulated by ET-1 although ETB receptors have been suggested to contribute to O_2- production.[51,52] ET-1 has been shown to increase the expression of NOX2, the rate-limiting subunit of NADPH oxidase.[56] The stimulating effect of ET-1 on O_2- production may also be coupled to the NADPH oxidase subunit p22phox.[56,57] These data are in agreement with the *in vivo* observations in transgenic mice over expressing ET-1.[58] These mice exhibit endothelial dysfunction, increased NADPH oxidase activity, and increased expression of NOX2. A recent report shows that ET-1 increases expression and activity of p47phox in rat aortic rings via the ETA receptor which would suggest that ET-1 is involved in the activation of NADPH oxidase.[59] It was recently demonstrated that the selective ETA antagonist avosentan significantly reduces aortic plaque formation in diabetic apoE−/− mice, independently of effects on blood pressure, lipid, or glucose levels. The anti-atherosclerotic effect of avosentan was associated with a significant reduction in macrophage infiltration and reduced nitrotyrosine levels, reflecting a parallel decrease in oxidative stress and AS.[60] This observation supports the notion that ET-1-mediated stimulation of oxidative stress is of importance, although the link between increased oxidative stress and AS is complex, as exemplified by the observation that genetic deletion of p47phox, an essential component of NADPH oxidase, did not affect the progression of AS in apoE−/− mouse model.[61] On the other hand, O_2− generation may increase AS by activating mitogenic signalling pathways in VSM cells.[62]

The vasoconstrictor effects of ET-1 may be more pronounced in states of reduced bioavailability of the eNOS co-factor tetrahydrobiopterin (BH4).[63] Recent data demonstrate that ET-1 mediates O_2- production and vasoconstriction through activation of NADPH oxidase and uncoupled NOS in the rat aorta.[54] The uncoupling of NOS means that NOS generates O_2- instead of NO in states of BH4 deficiency. These effects could be inhibited by BH4 and by dual ET receptor blockade, but not by selective ETA receptor blockade.[54] ET-1 may also promote BH4 deficiency in a rat model of hypertension via an ETA-mediated NADPH oxidase pathway, which contributes to impaired endothelium-dependent relaxation.[64]

ET-1 has been demonstrated to be associated with increased oxidative stress and endothelial dysfunction in humans. ET-1 mediates a marked increase in O_2- production in internal mammary arteries and saphenous veins from patients with CAD via a mechanism involving a flavin-dependent enzyme which is likely to be NADPH oxidase also in humans.[65] ET-1 also stimulates O_2- formation and impairs endothelium-dependent vasodilatation

in human venous bypass conduits from patients with CAD and diabetes.[66] The impairment in endothelium-dependent vasodilatation *in vivo* induced by ET-1 in healthy humans can be prevented by administration of the anti-oxidant vitamin C.[67] Conversely, ET-1 is increased in human CAD by oxygen-derived radicals *ex vivo* and *in vivo*,[68] indicating a vicious circle of oxidative stress leading to increased expression of ET-1 which, in turn, increases oxidative stress. Taken together, these data suggest that increased oxidative stress induced by ET-1 in the vessel wall contributes to endothelial dysfunction that, together with pro-inflammatory effects, may be important mechanisms behind development of AS.

PRO-INFLAMMATORY EFFECTS OF ET-1

Apart from its direct vasomotor activity, ET-1 has been implicated in the inflammatory processes within the vascular wall. Specifically, ET-1 activates macrophages, resulting in the release of pro-inflammatory and chemotactic mediators, including TNF-α, IL-1, IL-6, and IL-8.[69-71] Cardiac overexpression of ET-1 in mice is associated with an inflammatory response involving increased activation of the pro-inflammatory transcription factor NF-κB and expression of several pro-inflammatory cytokines including TNF-α, IL-1, and IL-6.[72] In turn, transcription factors and pro-inflammatory cytokines such as NF-κB, TNF-α, and IL-6 stimulate ET-1 production.[73] ET-1 enhances the expression of adhesion molecules on TNF-α stimulated vascular endothelial cells[74] suggesting an involvement of ETB receptors. Furthermore, ET-1 stimulates aggregation of polymorphonuclear neutrophils.[75] Conversely, ET receptor blockade attenuates the accumulation of neutrophils and myeloperoxidase activity in the ischaemic myocardium.[76] Although not a true AS model, it has been shown that vascular inflammation and neointima formation following vascular injury by carotid artery ligation is attenuated in endothelial cell ET-1 knockout mice.[77]

IL-6 has been implicated in the development of AS[78] and endothelial dysfunction in humans.[79] As noted above, ET-1 stimulates IL-6 release *in vitro* and *in vivo*. The release of IL-6 induced by ET-1 from human VSM involves activation of NF-κB. Possibly, release of IL-6 may further increase oxidative stress as suggested by the *in vitro* observation that IL-6 induces production of ROS.[80]

ET-1 RECEPTOR ANTAGONISM THERAPEUTIC APPROACH

Need for a Cause-Based Therapeutic Strategy

It has been shown that it is essential to focus and act on the risk factors associated with PAD, since PAD patients are at high-risk of CV morbidity and mortality. Between 2-4% of patients with IC present a non-fatal CV event during the first year of diagnosis, and approximately 60% will die from various CV causes.[81,82] For patients with critical ischaemia, the prognosis is even worse, with higher rates of limb loss and mortality from CV causes. Therefore, treatments for this condition must be directed, on one hand, towards treating the CV risk factors and, on the other hand, to treat localised symptoms caused by the disease.

25% of IC patients progressively deteriorate, requiring intervention in 5% of all cases in the form of revascularisation of the lower limbs, with 1-2% requiring a major amputation.[83,84] Current treatment options in this regard include smoking cessation - a dose-dependent relationship between smoking and the severity of PAD has already been demonstrated[85] - and the promotion of exercise programs for patients with IC, since exercise, in addition to attenuating CV risk factors, has also been shown to relieve the symptoms of the disease.[86,87] On the other hand, structured supervised exercise programmes have been shown to be effective in improving a patient's walking ability.[88] However, these programmes are not available in most health systems as their high cost and low compliance rate make them prohibitive and difficult to implement. Intermittent pneumatic compression therapy has also been shown to be effective in alleviating disease symptoms.[85]

With respect to surgery, endovascular revascularisation, open surgery, and other techniques have proven effective, and remain the best options when trying to save a limb and improve quality of life (QoL) in critical ischaemia. It should be noted, however, that there is still much controversy surrounding the relevance of surgical indications in patients with claudication, with some contending that surgery should be reserved for cases of critical ischaemia. The results of revascularisation techniques depend on the location and morphology of the lesion, but in a significant percentage of cases it cannot be carried out due to an associated comorbidity or a recurrence

of the symptomatology over time as a result of vessel restenosis. Thus, percutaneous transluminal angioplasty (PTA) has been shown to improve QoL at 3 months in patients with IC.[89] However, up to 60% of patients develop restenosis, with a non-negligible recurrence-of-symptoms ratio. On the other hand, applied pharmacological therapies have shown controversial benefits. While such therapies are generally employed to manage limb pain and improve QoL for patients, none has shown any convincing efficacy in preventing amputation.[82] Clinical trials have evaluated numerous drug therapies, such as naftidrofuryl, pentoxifylline, L-carnitine, levocarnitine, garlic, testosterone, cilostazol, and chelation therapy, but none have proven effective or less effective than standard treatments.[90,91]

At present, ET receptor antagonists are approved for the treatment of pulmonary arterial hypertension and for the prevention of new digital ulcers in systemic sclerosis. Accumulating evidence suggest that ET-1 is of pathophysiological importance in the development of several CV diseases including AS, PAD, and diabetic angiopathy. The expression of ET-1 and its receptors are markedly altered during disease progression, resulting in increased biological importance of the ET-1 system. Results from small randomised clinical studies support data from promising initial pilot studies. Considering the potentially important role of ET-1 in the development of vascular dysfunction reviewed in the present article – conditions with increased inflammatory activity, oxidative stress, and vascular tone such as AS and PAD – larger clinical trials using ET receptor antagonists are encouraged and needed.

Preclinical Studies with ET Receptor Antagonists

A dual ETA/ETB receptor antagonist reduced foam cell formation in macrophages exposed to oxidised LDL.[92] In the same study, Babaei et al.[92] showed that the ET receptor antagonist significantly inhibited the development of AS in LDL receptor knockout mice. These data clearly suggest that ET-1 is involved in the development of AS and that ET receptor blockade exerts anti-atherogenic effects.

CONCLUSION

In summary, there is evidence that the ET-1 pathway is involved in PAD with raised plasma levels and local sources of ET-1. In the diseased arteries, ET-1 is likely to play a role in atherogenesis. More recent evidence of a potential role of ET-1 in ischaemia-induced skeletal muscle damage suggests that this may be a more useful target for treatment. ET antagonism may play an adjunctive role in improving microvessel function and reducing tissue damage within the affected muscle. However, in patients with advanced atherosclerotic lesions, manipulation of the ET-1 pathway is unlikely to be of significant benefit in terms of lesion regression and improving blood flow. Results from small randomised clinical studies support data from promising initial pilot studies. Considering the potentially important role of ET-1 in the development of vascular dysfunction reviewed in the present article — conditions with increased inflammatory activity, oxidative stress, and vascular tone such as AS and PAD — larger clinical trials using ET receptor antagonists are encouraged and needed.

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