REVIEW ARTICLE

Novel therapeutic targets for preserving a healthy endothelium: Strategies for reducing the risk of vascular and cardiovascular disease

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Abstract

The endothelium lies in a strategic anatomical position between the circulating blood and the vascular smooth-muscle cells. It is a source of vasodilators such as nitric oxide, prostacyclin, and hyperpolarizing factor as well as heparin-like substances and other molecules with anti-proliferative properties. These effects of endothelial cells may explain why platelets and monocytes usually do not adhere at the blood vessel wall. However, under pathological conditions, endothelial dysfunction occurs and significantly contributes to the increase of platelet--vessel wall interaction, vasoconstriction, pro-inflammation, and proliferation. Under these conditions, endothelium-dependent vasodilation is reduced, and endothelium-dependent constrictor responses are augmented. Upon vessel wall injury, the platelets rapidly adhere to the exposed sub-endothelial matrix, which is mediated by several cellular receptors present on platelets or endothelial cells and various adhesive proteins. Subsequent platelet activation results in the recruitment of additional platelets and the generation of platelet aggregates, so forming a stable platelet plug. Therapeutic strategies aimed at improving or preserving endothelial function therefore may be promising in terms of preventing and treating coronary artery disease. Diagnostic modalities for assessing endothelial function should allow for the early detection of vascular endothelial dysfunction before the manifestation of serious adverse vascular disorders. (Cardiol J 2011; 18, 4: 352–363)

Key words: vascular, endothelial cell, cardiovascular disorders, coronary syndrome, vasoconstriction, vasodilation, inflammation, anti-inflammation, anti-platelet, pro-platelet, anti-coagulant, pro-coagulant, diagnosis of endothelial function

Introduction

Cardiovascular disease (CVD) as a result of atherosclerosis is a well-known and significant contributor to morbidity and mortality worldwide, affecting 80 million people aged 20 and over in the United States in 2006. In the United States in 2006, approximately 7,095,000 inpatient cardiovascular operations and procedures were performed. The estimated cost (direct and indirect) in the US for CVD in 2009 was $475.3 billion. Furthermore, increased risk is associated with advancing age; in

Table 1. Types of anti- and pro-atherothrombogenic molecules in endothelium (adapted from Blann et al. [2]).

	Anti-atherothrombogenic mediators	Pro-atherothrombogenic mediators
Cell surface molecules	Thrombomodulin Protein C Heparin and heparin sulfate Ectonucleotidases Tissue factor pathway inhibitor Endothelial estrogen receptor-alpha (ERα)*	Tissue factor Binding sites for coagulation factors
Released molecules	Nitric oxide Prostacyclin Tissue plasminogen activator (t-PA) t-PA binding sites	Thromboxane A2 Platelet activating factor Von Willebrand factor Endothelin

*ERα plays a key role in the E2-induced prevention of endothelial dysfunction after ischemia/reperfusion [60]

2005, more than 150,000 Americans who died from CVD were younger than 65 [1]. Given an average US life expectancy of about 77 years, these deaths are deemed premature, and necessitate advancements in the treatment and prevention of CVD.

Among the 80 million people aged 20 plus in the US affected by CVD from atherosclerosis, peripheral arterial disease affects about 8 million of them and has risk factors similar to those of coronary artery disease [1], probably due to homologous origins of disease, further underscoring the need to address the notion of endothelial cell dysfunction — the apparent source of CVD. Major advances have been made in understanding the early mechanisms of endothelial dysfunction. Given the complexity of the genetics, molecular mechanics, and physiology of this disease, it is important to continue to pursue a deeper understanding of its intricacies. Such research will likely lead to prevention strategies as early in the disease process as possible. This would be a far more cost-effective alternative to treating already established CVD. Certainly, any advances in treatment and prevention strategies against CVD will greatly benefit our healthcare system.

Endothelial cell dysfunction is a hallmark of the early development of atherosclerosis. While many medications that reduce the risk of CVD exist, including statins, purified fish oils, ACEIs/ARBs, and thiazide diuretics, "all the major risk factors for atherosclerosis have been associated with impaired nitric oxide activity," [2] which is a primary marker for endothelium dysfunction [3, 4].

This, combined with the rise of flow-mediated dilation as an indicator of a patient's nitric oxide (NO) bioactivity, can open the door to identifying patients with early endothelium dysfunction [4]. These patients would likely be more susceptible to various forms of CVD, so early identification is highly beneficial. This practice can pave the way to testing a growing array of medications aimed at correcting early endothelial cell dysfunction. It is all but certain that such correction will lead to longer term risk reduction and improved clinical outcomes.

Reduced bioactivity of NO is the current benchmark for recognizing endothelial cell dysfunction [3]. However, despite NO's predictive value, many other biological molecules are responsible for endothelial cell homeostasis, giving a wide variety of potential drug targets (Fig. 1). Identifying and understanding key biological markers/mediators involved in the pathophysiology behind endothelium dysfunction should ultimately lead to methods of treating or preventing the early onset of atherogenesis. The literature shows that endothelial cell dysfunction and plaque formation is characterized by a balance shift featuring decreased vasodilatory, thrombolytic, anti-inflammatory, and anti-coagulant molecules (of which NO is arguably the most significant) relative to their pro-coagulant, vasoconstrictive, and thrombotic counterparts [3].

This review is a summary of some potential drug targets geared toward stimulating the anti-atherogenic properties of the endothelium, centered on some of the key biological functions of the endothelial cell. Table 1, adapted from Blann et al. [2], identifies key components of the endothelium, some of which are discussed in this review. In addition, there are other molecules that are pro-atherogenic and are not directly related to the endothelium (such as angiotensin II, thrombin, plasmin, and fibrin) that are not listed in Table 1. The anti-atherogenic column in Table 1 lists molecules that are of an anti-coagulant, anti-thrombotic, anti-inflammatory, anti-adhesive, or vasodilatory nature. The opposite is true of the pro-atherogenic column. The molecules in either column should not be considered healthy, or unhealthy, because pathologi-
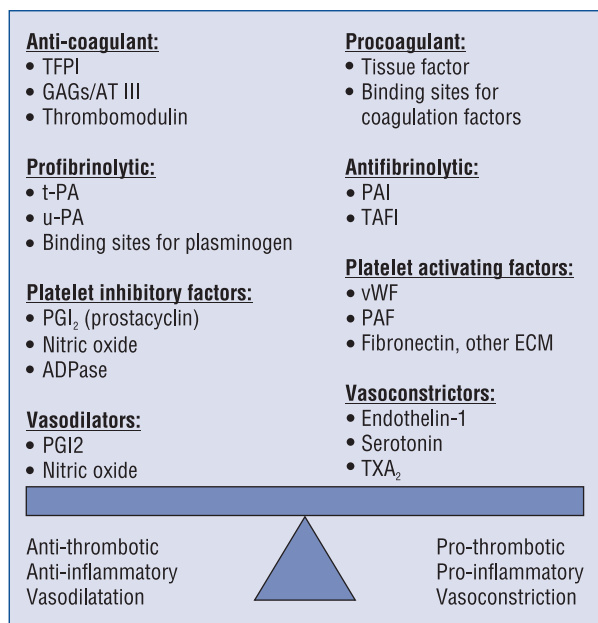
Figure 1. Hemostasis of vascular endothelium: endothelium as an organ represents $1–6 \times 10^{13}$ cells monolayer, weighs 1 kg, covers six tennis courts, and maintains hemostasis of various physiological functions. Modulator of vascular tone via nitric oxide, prostaglandin I_2 (PGI$_2$, prostacyclin), endothelin and various receptors. Regulator of hemostasis via anti-thrombotic, pro-thrombotic mediators. Modulator of inflammation via pro- and anti-inflammatory mediators.

cal conditions tend to arise only from irregular expression of these molecules. It is important to note that factors in the 'pro-atherogenic' category are of great physiologic importance for normal health, and that complete inhibition of any of these factors will likely have adverse results. However, these factors, in certain cascades, can play atherogenic roles in a dysfunctional endothelium. Furthermore, some anti-atherogenic molecules, which lyse clots, may play a role in triggering thrombosis in advanced lesions.

Organic nitrates and innovative nitric oxide donors for the endothelium

Organic nitrates such as isosorbide dinitrate (ISDN) or isosorbide mononitrate (ISMN) are commonly used in the treatment of stable angina and the improvement of exercise tolerance in the clinical setting. However, little attention has been paid to the great potential that may be derived from recent advances in the understanding of nitrate function. For example, nitrates have been identified that

can increase NO production, and perhaps slow or prevent the progression of atherosclerosis, unlike traditional nitrates used for stable angina. Such molecules may be important given the fact that routine administration of organic nitrates is typically associated with tachyphylaxis, decreased NO bioavailability, and the development of endothelial cell dysfunction [5]. This is typically caused by the generation of reactive oxygen species (ROS), generally produced by the resultant superoxide anion from the nitrate metabolic pathway [6, 7]. ROS inhibit mitochondrial aldehyde dehydrogenase (ALDH-2), which is a primary enzyme for the metabolism of certain nitrates. Consequently, long term repetitive treatment with nitroglycerin, *versus* ISMN and ISDN, is more associated with worsened endothelial cell-dependent vasodilation and a decrease in NO bioavailability [5, 7]. However, among all organic nitrates, repeated long term administration is still linked to nitrate tolerance.

While nitrate-free intervals generally decrease or prevent long term nitrate tolerance [7], the control of ROS is still of significant pharmacologic importance, and has been a focus of recent research regarding organic nitrate function. Compounds possessing three nitrate groups go through a mitochondrial biotransformation that is not undergone by compounds with two or fewer nitrate groups. This biotransformation is associated with the increased generation of ROS [6].

This has led to ISMN and ISDN being preferred in clinical practice for the long-term treatment of stable angina since they do not seem to significantly stimulate the production of ROS [6]. This is likely due to their metabolism being through the non-mitochondrial 'low affinity pathway' in tissues [8]. However, Dragoni et al. [6] have recently shown that pentaerithrityl tetranitrate, typically avoided due to its mitochondrial metabolism, exhibits an additional antioxidant effect and an ability to stimulate protective genes that code for the generation of heme-oxygenase-1 and ferritin. The authors hypothesize that these genes play a role in the establishment of endothelial cell 'pre-conditioning' [6]. Pre-conditioning means that tissues, such as the myocardium, undergoing transient periods of ischemia, can become resistant to periods of severe ischemia. This phenomenon was shown to be consistent in the endothelium by Zahler et al. [9], and is another area of great interest and therapeutic potential.

On a similar front, dietary nitrite is making a return appearance. Although in the past nitrites have been associated with negative consequences,

a recent extensive report showed the presence of many beneficial effects of nitrites on the endothelium [10]. In rats with micro-vascular inflammation induced by a high cholesterol diet, nitrite supplementation in water proved protective against endothelial dysfunction. Nitrite ingestion is involved in returning C-reactive protein levels back to normal in these mice and the preservation of BH_4, a redox enzyme that is involved in NO formation. Nitrite is also known to be depleted in hyper-cholesterolemic states. Given this, and the abundance of nitrite in vegetables (where they are associated with 'heart health'), and given the hypothesis that N-nitrosamine formation is the true carcinogenic worry, a re-evaluation of nitrite supplementation seems sensible [10]. Perhaps nitrite given as a 'cocktail' with compounds that inhibit N-nitrosamine formation could provide long-term protection of the endothelium without adverse consequences.

Thrombomodulin

Thrombomodulin (TM) is a 100 kDa membrane protein that is expressed on the surface of endothelial cells. It is involved in maintaining the balance between the anti-coagulant and pro-coagulant properties of the endothelium [11, 12]. Each endothelial cell typically contains 50,000–100,000 TM molecules [13]. TM possesses a large number of anti-atherogenic functions. Among these are its activation of anti-coagulant protein C via formation of the thrombin-TM complex, and its facilitation of thrombin–anti-thrombin complex formation via its chondroitin sulfate moiety [14, 15]. TM is also responsible for the activation of thrombin activatable fibrinolysis factor which is also being examined for its anti-inflammatory properties [15]. TM is a multidomain peptide that can be recombinantly expressed in various forms with a large variety of functional activities [16, 17]. Given TM's wide range of anti-coagulant functions, it is unsurprising that local TM deficiencies have been linked to thrombosis and atherogenesis in animal models [14]. The functions of TM, and the physiologic consequences of localized TM deficiency, clearly underscore its role in the prevention of plaque formation. [14]. In addition to this, TM is sub-normally expressed in endothelial cells lining atherosclerotic plaques [14, 18], and loss of TM activity in vein grafts is linked to early thrombosis [19].

Given this information underscoring the physiological consequences of lacking TM, the obvious follow-up question is: what would be the result of hyper-expression of TM along cells in the endo-thelium? Furthermore, since TM is less prevalent in endothelium lining atherosclerotic plaques, the next question is: what is TM's role in the stabilization of plaques? While treatment of atherosclerotic plaques would ideally involve reversal and removal of the fatty streak, perhaps stabilizing a slightly occluded vessel could be a less invasive approach to preventing plaque growth and minimizing the risk of occlusive thrombosis.

Many genes have been identified as associated with changes in TM function and therefore clinical manifestations of disease. These genetic polymorphisms tend to be associated with an increased risk of myocardial infarction or atherosclerosis, and are listed in Weiler and Isermann [14]. These clinical correlations match up with the notion that loss of TM or inactivation of endothelial TM can lead to vascular complications. Loss of TM expression on the endothelial cell membrane can occur via multiple mechanisms. These include oxidation of functional domains of TM, any trigger of large amounts of cytokines — generally from sufficient endothelial cell injury — which in turn suppress its gene transcription, or neutrophil-triggered release of soluble TM [14, 15].

These facts refine the previous questions: would increased cellular TM expression make the endothelium more resistant to these inflammatory response cascades? Would such hyper-expression of TM lead to a more non-reactive endothelium that is more resistant to the development of atherosclerotic lesions? Or, would this lead to the endothelium having a decreased ability to repair vascular injury? Furthermore, this information underscores the need to identify the genetic targets that control TM expression, so that they can be modified in order to design experiments to answer these questions.

In 2004, a new gene transfer method using a "ligand-facilitated transfer of the cationic liposome: TM gene complex" was used to induce the over-expression of TM in rat inferior vena cavas [19]. This over-expression was induced prior to excision and vein graft operation. Amazingly, under reduced-flow conditions of venous endothelium, over-expression of thrombomodulin was achieved by this high-efficiency method. This translated to a greater thrombo-resistance (i.e. less clotting induced by thrombin) in the grafts with over-expressed TM *versus* control groups, thus preventing acute graft failure [19]. These results, combined with full length TM's ability to inhibit smooth muscle cell proliferation, make it a good candidate for *in vivo* studies related to operative occlusion prophylaxis [20]. TM over-expression can perhaps protect

against stent failure and a variety of graft failures. While experimentation is currently focused on venular endothelium TM, the advent of even higher efficiency gene transfer techniques (potentially used under normal flow conditions) could open up a world of clinical opportunities with regard to arterial endothelium TM.

Soluble thrombomodulin is another interesting factor with potential for controlling atherogenesis/thrombosis. Soluble TM appears to be a cleaved fragment of the membrane TM that is released into the bloodstream via 'neutrophil-elastase-dependent proteolytic release' [13, 14]. Soluble TM is known to be elevated in a variety of disease states associated with endothelial cell injury [21]. This form of TM still retains significant amounts of cofactor/biologic activity relative to cellular TM. Soluble TM still has the ability to inhibit fibrinolysis, and some fragments retain the ability to activate protein C at 30–50% of the affinity of cellular TM. Variations in the types and sizes of soluble TM fragments also seem to produce drastic differences in functional activity [21]. This variability is likely due to an array of expressions of its chondroitin sulfate (CS) moiety among fragments [16]. The chondroitin sulfate moiety is responsible for increasing TM's ability to inhibit thrombin, and furthers the binding of anti-thrombin III to thrombin. TM possessing the chondroitin sulfate moiety (at full length) is more prevalent in arterial endothelial cells than in venous endothelial cells [16]. Another example of different functional fragment activity involves the lectin domain. The lectin-like domain is associated with anti-inflammatory properties, while showing no anti-coagulant or fibrinolytic actions. It has been shown to protect endothelial cells in certain destructive conditions, and disrupts monocyte adhesion to activated endothelium [14, 22].

Several different soluble TM fragments have been identified, found in different concentrations among different cardiac risk disease states [21]. Isolating and understanding the different structure--function relationships among soluble TM fragments could lead to the identification of the most therapeutically beneficial components for correlating disease states. For instance, it is important to identify the beneficial or adverse effects of routine administration of rTM consisting of only the lectin--like domain. Thus far, it has been shown to reduce inflammation in mouse models, and seems to hold promise in various inflammatory disorders, including arthritis [22]. Perhaps these anti-inflammatory properties could provide long-term risk reduction by reducing micro-vascular inflammation in CVD.

Other manipulations could provide a safer and more specific alternative for anti-coagulant therapies in the setting of acute coronary syndromes. While the current paradigm is centered on unfractionated and low molecular weight heparins, bleeding complications are still of significant concern. However, manipulated soluble TM fragments can be explored as a potentially safer and comparably efficacious alternative. For example, the soluble--TM-thrombin complex still exhibits the anti-coagulant property of protein C activation. In addition, some soluble and recombinant TM does not have the chondroitin sulfate moiety, which directly interferes with its abilities to activate protein C and facilitate the formation of the thrombin–anti-thrombin complex [14, 16]. Perhaps these fragments can provide a therapeutically beneficial balance of activity. Different concentrations of these fragments may exhibit safer degrees of anti-coagulation while maintaining efficacy.

In short, purifying and experimenting with various TM domain fragments may unlock an entirely new class of acute care blood thinners or anti-inflammatory agents.

Tissue factor and tissue factor pathway inhibitor

Tissue factor is a transmembrane protein that plays a significant role in the extrinsic pathway of the coagulation cascade. It is expressed on various immune cells and smooth muscle cells, and certain conditions cause it to be expressed in endothelial cells. Tissue factor is abundant in atherosclerotic lesions, and is associated with an increased risk of thrombus formation within those lesions. It is regulated by tissue factor pathway inhibitor (TFPI), which exerts its effects via factor Xa inhibition, and is predominantly seen in the endothelial cells of the microvasculature [23].

There are a variety of TFPIs, and one of the most clinically promising versions is active-site--inactivated recombinant factor VIIa. Its mechanism of action is competitive inhibition of factor VIIa to prevent binding to tissue factor. Inhibition of thrombosis without prolonged bleeding time has been observed in animal models using this inhibitor [23]. Additionally, monoclonal antibodies against tissue factor provide a variety of beneficial effects, including reduction in thrombin-mediated inflammation [23].

A recent trial has demonstrated that thrombin is a key molecule for initiating tissue factor in human umbilical vein endothelial cells. This mechanism is further enhanced by plasmin treatment. The

study concluded that plasmin's proteolytic activity was needed to create this increased expression of tissue factor. While other explanations are possible, it is likely that plasmin increases local tissue factor by cleaving TFPI [24]. Should this be true, the next question is whether an elevated level of TFPI would be completely degraded by the same level of enzyme activity, or if plasmin's activity is of limited capacity.

It appears that plasmin activity can be overridden, since recombinant TFPI inhibits thrombus formation and fibrin deposition in injured arterial segments (two events dependent on thrombin activating TF) [23]. Increased TF activity in atherosclerotic plaques is associated with conditions more conducive to thrombosis [23]. Perhaps increased TFPI levels locally can provide protection against thrombus formation when performing stent operations. This opens the door to therapeutic options similar to those of thrombomodulin. Perhaps high-efficiency gene transfer over-expression of TFPI in athero-prone segments of endothelium can correlate with better clinical outcomes during stenting or angioplasty.

With regard to tissue factor itself, some tissue factor polymorphisms are associated with poor clinical outcomes in CVD. The +5466A>G polymorphism has been shown to influence not only the risk of cardiac death, but also the effect of statins on thrombin formation [25]. Undas et al. [25] identified a certain polymorphism that may be linked to variable pleiotropic effects in statins, aside from cholesterol lowering. This study is the first step on a path toward potentially identifying optimum drug regimens based on patient genotype, and may be important in the setting of personalized medicine approaches in the future by aiding the risk stratification of patients. Further identifying and characterizing high risk polymorphisms with regard to factors related to the endothelium will be crucial in the design of new patient-specific therapies. Tissue factor, and its pathway inhibitor, is certainly of interest for future pharmacogenomic approaches.

Endothelial lipase and HDL modulation by tissue factor pathway inhibitor

Endothelial lipase (EL) is an intriguing lipid hydrolyzing enzyme that robustly influences high density lipoprotein (HDL) metabolism [26], but the health implications of this modulating activity in humans are unknown. It is the most recently discovered member of the lipase gene family that includes lipoprotein lipase and hepatic lipase. In contrast to those other two lipases, EL is produced by endothelial cells. EL activity is increased in the midst of inflammatory states as reflected by its upregulation by proinflammatory cytokines. It is interesting that EL has been found to hydrolyze HDL, and that EL activity may explain the inverse relationship that exists between inflammatory states and levels of HDL [27]. Although it is possible that downregulating EL will lead to increased HDL and reduced risk for CVD, the health implications of modulating EL are unknown. However, if a therapeutic agent reduces EL and inflammatory cytokines while increasing HDL, this would suggest that the agent possesses strong therapeutic potential. The benefits of such metabolic regulation by this novel agent await investigation of the cardiovascular effects on humans in states of health and disease.

Prostacyclin

Prostacyclin is another molecule endogenous to the endothelium that is responsible for vasodilatory and protective actions on the endothelium. Inhibitors of prostacyclin formation or activity will likely inhibit normal endothelial function, and consequently open the door to atherosclerotic plaque formation [28]. Increased oxidative stress via superoxide anion formation leads to peroxynitrate formation [8]. Peroxynitrate is known to inhibit prostacyclin synthase (PGI-S) [28]. Given that prostacyclin is vital in controlling vasodilation and inhibiting thrombus formation [3, 29], this suggests a mechanism for how oxidative stress can make the endothelium more prone to developing atherosclerotic lesions. In addition, the notion that various lipid peroxides inhibit PGI-S further supports the role of decreased prostacyclin protection in atherosclerosis [28], because oxidant injury is linked to both lipid peroxide formation and atherosclerosis [30].

The role of prostacyclin has been established as vital to a healthy endothelium, and so the development of prostacyclin analogs followed. Prostacyclin analogs have already shown degrees of efficacy in disease states including pulmonary arterial hypertension, Buerger's disease, and critical limb ischemia [28, 31]. More recently, it has been shown that iloprost, a prostacyclin analog, has the ability to increase circulating endothelial progenitor cells (EPCs) in patients with critical limb ischemia [31]. Following up on data from previous studies showing improvement in ulcer healing, level of pain, and delayed amputation, the authors decided to uncover the potential mechanism behind these clinical benefits. Based on the evidence that iloprost can

activate many pro-angiogenic genes, and that clinical benefits in critical limb ischemia come from more than anti-platelet and vasodilatory actions, the authors set out to determine iloprost's effect on EPCs. The dosing rate for the IV infusion was a median of 1.2 ng/kg/min for six hours to prevent hypotension and tachycardia. Of the 23 enrolled patients, 14 had reduced pain and the other nine had stabilized pain. EPC levels were increased from a median of 13,812/mL pre-treatment *versus* 23,739//mL post treatment. These results demonstrate that iloprost does seem to increase EPCs, an effect to which the authors attributed the positive clinical response as reflected by pain reduction and delayed time to amputation consistent with previous studies.

The authors acknowledged that the data is short term and may not correlate with long-term outcomes, and that the population sample was very small. However, given the data supporting the ability of EPCs to promote angiogenesis, longer term trials are warranted to clarify iloprost's benefit in a variety of vascular conditions [31]. Iloprost is also associated with good tolerability in recent trials involving limb ischemia [31, 32]. Concordantly, it seems appropriate to study tolerable doses of iloprost for angiogenic benefits in other patient populations at high risk for vascular disease. Future research may focus on the development of an oral or SQ formulation suitable for outpatient therapy, and investigations regarding that form of dosage would affect overall atherosclerotic plaque formations in high risk patients.

With the mechanistic potential for endothelium preservation, and this newfound efficacy data in peripheral ischemic disease, perhaps the next stage would be to identify the most tolerable analogs and test them in clinical trials. Circulating endothelial progenitor cells could indicate the severity of vascular disease. Increased EPCs of normal functional capacity correlate to a lower risk for developing atherosclerotic lesions, and EPCs are linked to the regeneration of new blood vessels [5, 31].

Moreover, work by He et al. [33] has demonstrated that the angiogenic properties of EPCs are dependent on the biosynthesis of prostacyclin. Characterizations of the functions of iloprost on EPC function have helped elucidate some of the mechanisms and pathways by which EPCs function to proliferate and promote angiogenesis [33]. Continuing research along the lines of prostacyclin analogs with regard to EPC function will likely lead to classification of more specific therapeutic targets in EPCs; this should ultimately lead to new drug classes for the prevention and treatment of atherosclerotic vascular diseases.

Ligustrazine derivatives

Recent publications have discussed various forms of ligustrazine derivatives as novel compounds for use in the protection of endothelial cells. While these are not endogenous compounds in the body, they deserve attention for anti-oxidant protection of the endothelium. The studies examined the effects of various derivatives of ligustrazine on ECV-304 and HUVEC cells. While ligustrazine has a wide variety of pharmacological uses, the compound has been more recently noted to have protective effects on injured vascular endothelial cells [34, 35].

The first study evaluated a variety of 2-acyloxymethyl-3,5,6-trimethylpyrazine derivatives. Some compounds exhibited the ability to protect HUVEC cells from hydrogen peroxide (oxidative stress) damage, while others stimulated the proliferation of normal cells while being unable to protect the cells from damage [35].

In the second study, ECV-304 cells were used instead of HUVECs. It was found that one particular derivative produced a greater protective effect against hydrogen peroxide damage of endothelial cells *versus* the other derivatives or original ligustrazine. The derivative, which included the bisphenylmethyl group on the methyl position of ligustrazine, provided the lowest EC50 for ECV-304 protection against hydrogen peroxide damage.

The third part of these experiments included acylpiperazinyl derivatives. 2-(4-salicyloyl-1-piperazinmethyl)-3,5,6-trimethylpyrazine (compound E33) was determined to have the most beneficial effect, again on ECV-304 cells exposed to hydrogen peroxide [36]. Structure-activity relationships are discussed in each of the respective articles, and biological assays are in progress to further understand the effects of these compounds. This is an interesting class of molecules derived from a traditional Chinese medicine herb, and may perhaps pave the way toward new agents in preserving the integrity and balance of the endothelium. Considering that compounds in each of the three studies were found to protect against oxidative stress, coupled with the physiological role of oxidative stress on atherogenesis and NO dysfunction, it is easy to see why these are attractive compounds.

Adiponectin

Shibata et al. [37] recently reviewed adiponectin and CVD. Adiponectin is a plasma protein derived from adipose tissue. It is typically found in human plasma at concentrations of 3–30 μg/mL. When this pro-

tein is inadequate in patients with existing cardiovascular conditions, the result is increased incidence of adverse events. For example, low plasma adiponectin concentration is found in patients with acute coronary syndrome, while higher levels are associated with decreased myocardial infarction in healthy males. Another example is that higher serum adiponectin levels in patients with peripheral arterial disease correlate with better maximum walking distances and other positive signs. Furthermore, weak association with better cardiovascular outcomes in end-stage renal patients was shown in a recent meta-analysis. Most clinical evidence still shows the clear cut benefit of higher adiponectin levels in general CVDs [37].

Despite its semi-controversial clinical benefits, adiponectin has shown some evidence in animal models with regard to endothelium preservation and reduced levels of atherosclerosis. Adiponectin has been shown to play roles in angiogenesis, EPC levels, NO production by endothelial cells, and in the prevention of endothelial apoptosis in *in vitro* models [37]. Adiponectin also plays a stabilizing role in the endothelium by decreasing the expression of certain cell adhesion molecules via NFκB suppression. Lastly, its role in endothelium health is further established by its ability to reduce reactive oxygen and reactive nitrogen species (thus preserving NO function) in endothelial cells.

Adiponectin exerts its effects apparently via AMP-activated protein kinase. Various forms of adiponectin translate to different physiological effects. Primarily, the high molecular weight form is responsible for endothelial cell functions, while the other trimer form exerts greater effects on myocytes [38].

Shear stress and its mechanical stimulation of genetic and cellular targets

There are many arguments in favor of the benefits of exercise to reduce atherosclerotic risk. The mechanism behind this is likely related to the concept of shear stress on the endothelium, and how it regulates the function of molecules related to endothelial cell homeostasis.

Shear stress is essentially the drag force of blood rushing parallel along the vessel wall. This force is crucial in maintaining the anti-atherogenic state of the endothelium. Shear stress also helps explain why only certain parts of the human vasculature are susceptible to atherosclerotic plaque formation via endothelial cell dysfunction. This susceptibility is from areas of low or oscillatory shear

stress that shifts the balance of the endothelium more toward a pro-atherogenic and pro-inflammatory state. Shear stress is associated with the regulation of genes that control endothelial nitric oxide synthetase (eNOS) and TM [39–41]. These two molecules play key roles in maintaining an anti-coagulant and anti-inflammatory state in the endothelium [15]. Additionally, areas of low shear stress are shown to have lower expression of eNOS and are vulnerable to the development of atherosclerosis [41, 42]. Two key transcription factors, Nrf2 and KLF2, have been identified as responsible for about 70% of the expression of endothelial cell genes induced by shear stress [40]. It has been suggested that developing inducers of these molecules may provide the ability to stimulate an anti-inflammatory/anti-coagulant state in areas of low or oscillatory shear stress that are normally prone to atherosclerosis [40, 41]. While the physiologic consequences of such inducers are still unknown, developing these compounds could lead to a greater understanding of the pathway, and perhaps to the development of clinically important compounds.

Sirt-1 genetic modulator

Other methods for regulating the expression of eNOS, and thereby increasing NO output, include examination of the Silent information regulator class of proteins (Sirt). Sirt-1 is a deactylating protein covered extensively in a recent review by Potente and Dimmeler [43], as a novel target for maintaining endothelial cell homeostasis. To summarize, this protein is responsible for the regulation of many transcriptional factors including Foxo1, p53, and nuclear factor LXR in hepatocytes. Most relevant to the topic we are discussing is its regulation of eNOS. Sirt-1 is thought to regulate eNOS expression based on some key pieces of evidence. The administration of resveratrol, which activates Sirt-1, has also been shown to increase the expression of eNOS [43]. In addition, Sirt-1 inhibition in the arterial endothelium inhibits endothelium-dependent vasodilation. It has been shown that Sirt-1 promotes the output of NO via the deacetylation of eNOS [44]. Furthermore, caloric restriction, which lowers blood pressure in healthy individuals, has been shown to promote the deacetylation of eNOS in mice. Putting this all together, Sirt-1 is a deactylating regulator of the expression of eNOS [43, 44]. Sirt-1 may also play roles in terms of vascular growth, removal of cholesterol from peripheral tissues, improvement of insulin sensitivity, and endothelial senescence [43].

Essentially, Sirt-1 can control risk factors across the board with respect to diabetes, high cholesterol, and aging effects in the body. On the downside (and potential upside), Sirt-1 activity is enhanced in certain tumors, which may be a pitfall to chronic administration of a Sirt-1 activator (yet paves the way for new anti-tumor agents). Consequently, it seems simultaneously attractive yet potentially hazardous as a drug target for disease [43]. While Sirt-1 activity suppresses cancer in breast and colon cells, it appears that Sirt-1 also is responsible for the maintenance of prostate cancer and certain epithelial cell cancers [45]. As noted by Kim and Um [45], a better understanding of Sirt-1's different mechanisms in these cancer cells *versus* its anti-aging properties would pave the way to a whole new avenue for targeting atherosclerosis and related chronic diseases.

Resolvins and lipoxins in endothelial dysfunction

Endothelial-leukocyte interactions trigger a novel and interesting set of inflammation-resolving, potent lipid mediators known as lipoxins and resolvins.

Lipoxins are metabolites of arachidonic acid, and resolvins are metabolites of the omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Through a mechanism that is not yet fully understood, they are known to bring an end to inflammatory processes occurring within the endothelium. Aspirin seems to play a key role in the intercellular interactions leading to the formation of these compounds [46]. For example, the formation of some lipoxins is mediated through aspirin acetylated PGHS-2 in endothelial cells and 5-lipoxygenase in leukocytes [47]. The role of lipoxins, in particular, appears to be centered upon the resolution phase of the inflammatory process, playing an inhibitory role on polymorphonuclear cell adhesion and chemotaxis. Lipoxins tend to inhibit formation of leukotriene B$_4$, a primary mediator of inflammatory cellular processes centered on recruitment of polymorphonuclear cells from postcapillary venules and their adhesion to endothelial cells [46, 48]. Furthermore, lipoxin-mediated enhancement of TGF-β serves as a cue to further promote the resolution of fibrosis [46].

During the later stages of the inflammatory process, signals from prostaglandins E2 and D2 trigger the resolution mechanism necessary for the recall of blood granulocytes. The mechanism consists of enhancing the formation of the omega-3 fatty

acid-derived resolvins through independent EPA and DHA pathways [49]. After an insult, these additional mediators allow for endothelium homeostasis to return. In a similar fashion to lipoxin production, resolvin formation is heavily influenced by the role of aspirin-mediated acetylation of the COX-2 enzyme [46, 50].

Aspirin-triggered lipoxins when used in an *in vivo* animal model promote inflammation resolution, apparently by shortening the maximal neutrophil number present during the inflammatory response. Resolvin E1 reduces the maximal neutrophil number and the time when this effect occurs [51]. Both lipoxins and resolvins seem to act through concomitant increase in the tissue repair and wound healing molecule TGF-β. Tissue repair and leukocyte infiltration is of key importance at the endothelial level, because macrophage incorporation and subsequent foam cell transformation plays a central role in the pathogenesis of atherosclerotic plaque formation [52]. Given this, it is believed that potent lipid mediators such as lipoxins and resolvins reduce the process of leukocyte rolling and transmigration and thus potentially attenuate the formation of atherosclerotic plaque on the vascular endothelium.

The role of lipoxins and resolvins has already been evaluated in apoprotein E-deficient mice with global leukocyte 12/15-lipoxygenase deficiency [53]. Expression of the 12/15-lipoxygenase was shown to be a protective mechanism against atherosclerosis, suggesting its lipoxin A4 and resolvin D1 products may be the mediators by which this protection is conferred. In the same study, treatment of human aortic endothelial cells with resolvin D1 led to a significant decrease in MCP-1 and IL-8, and a significant upregulation of the anti-inflammatory compound PDGF-β. Lipoxin A4 in turn was found to downregulate p-selectin expression. In another study [54], diminution of leukocyte transmigration was also found in choroid-retinal endothelial cell and leukocyte co-cultures, where addition of resolvin E1 and resolvin D1 not only decreased the number of leukocyte transmigrating past the endothelial layer, but also diminished IL-8, protein 1β and TNF-α.

Lastly, thrombus formation after endothelial plaque rupture could potentially be attenuated by these compounds. Using intravital microscopy and a murine animal model, Dona et al. [55] showed that *in vivo* administration of resolvin effectively reduces leukocyte rolling by 40%. It was also reported that resolvin E1 inhibits ADP and U46619 activated platelet aggregation in a concentration-dependent manner. These effects are indicative of re-

solvin's ability to reduce platelet aggregation and leukocyte-specific biochemical and structural processes that allow for homing, rolling, and migration across the endothelium.

Research suggests that resolvin E1 binds with high affinity to the G-protein coupled chemerin receptor (ChemR23) [56, 57] present in monocytes, macrophages, dendritic and endothelial cells. Despite recent evidence indicating the presence of ChemR23 receptors on endothelial cells [58], to date no studies have been published directly investigating the relationship between resolvins and Chem23 receptors or their effects on the endothelium. In other cells, RvE1 binding counter-regulates TNF-α-stimulated NF-κB activation and enhances macrophage phagocytosis of polymorphonuclear cells via Akt and ribosomal subunit rS6 downstream phosphorylation [59]. Lower binding affinity has also been reported between resolvins and the BLT-1 (leukotriene B4 receptor 1) present on neutrophils. It is possible, however, that these potent lipid mediators act through a series of receptors and pathways, and that additional targets remain to be identified on the endothelial cell surface.

Taken together, these findings suggest that atherosclerotic plaque formation is affected by a failure of the inflammation resolution mechanism through which potent lipid mediators act on vascular endothelial cells to achieve homeostasis. Potent lipid mediators, such as resolvins and lipoxins, may serve as potential novel therapeutic agents to attenuate ongoing inflammatory processes within the vascular endothelium. Even more intriguingly, exogenous administration of these and similar potent lipid mediators may even work as alternatives for individuals unable to obtain biochemical benefit from aspirin's anti-platelet effects. It could also be that the ingestion of EPA and DHA will lead to a sufficient quantity of the potent lipid mediators required for the activation of these mechanisms, at least in most individuals at high risk for CVD.

Diagnostic modalities for assessment of endothelial function might include local vasodilation by venous occlusion plethysmography, flow-mediated dilatation, arterial pulse wave analysis, pulse amplitude tonometry, microvascular bloodflow by laser Doppler flowmetry, biochemical markers, measurement of endothelial-derived microparticles progenitor cells, and glycocalyx measurements. These should advance the early detection of vascular endothelial dysfunction before the manifestation of serious adverse vascular or cardiovascular disorders.

Conclusions

The stage is set for the rise of novel, long-term risk reduction CVD therapies with regard to the endothelium. Nitrates with supplementary anti-oxidant effects such as pentaerithrityl tetranitrate are likely ready for human safety trials, and dietary nitrite may also be deserving of such research. These are basic ways of restoring some NO activity, and this logically would have a good chance of reducing the occurrence of long-term CVD development.

With regard to thrombomodulin, devising a method for localized expression would open the door to a much greater understanding of its ability as a potential therapy. Again, these attempts may include higher efficiency gene transfer methods, or even less invasive forms of creating reduced flow conditions, i.e. a meshwork (to slow flow) angioplasty device which at the peak of inflation releases a TM gene transfer vehicle. In addition, functional assays and subsequent clinical testing of various soluble thrombomodulin fragments just may open the door to a superior breed of anti-coagulant molecules. TFPI also presents itself as a possible player in this respect. Given the significant risks of thrombosis and re-occlusion in current angioplasty techniques, TFPI can have a role in localized reduction in risk of intimal thickening [23]. Perhaps stents secreting TFPI can prevent the initial TF cascade from inducing smooth muscle cell proliferation, and therefore reduce the long-term re-occlusion risk.

The idea of exploring the clinical potential of long-term low dose prostacyclin analogs seems plausible as well. Just as ACEIs and ARBs are used to protect the vasculature and renal system in hypertensive states, perhaps prostacyclin can be a well-tolerated protector of those with impaired endothelial function. These analogs, along with ligustrazine derivatives, may turn out to be molecules of great tolerability that can be used as a long-term hedge against accelerated endothelial cell dysfunction in high risk patients. Furthermore, the evidence is growing for the direct clinical utility of iloprost in the settings of critical limb ischemia, warranting larger and more controlled trials to assess its efficacy and tolerability.

The elucidation of further drug targets such as Nrf2, KLF2, and Sirt-1 opens the door for new compounds to be created as agonists or antagonists of these factors. Animal experimentation involving chemical modulation of these entities may shed further light on the favorability of these pathways for human clinical applications.

This review is an outline of some emerging therapies centered mainly upon the preservation of endothelial cell function. With scientific efforts continually plowing forward, perhaps CVD therapy will soon be revolutionized. In time, CVD may cease to be looked at as a treatable condition that is identified after much of its damage is done, but instead as a syndrome preventable through the stabilization of the endothelium.

Acknowledgements

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