Nitric oxide: a key factor behind the dysfunctionality of endothelial progenitor cells in diabetes mellitus type-2

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Abstract

Diabetes mellitus type-2 (DM-2) contributes to atherogenesis by inducing endothelial cell injury and dysfunction. Endothelial progenitor cells (EPCs) are essential to blood vessel formation, can differentiate into mature endothelial cells, and promote the repair of damaged endothelium. In DM-2, the circulating EPC count is low and their functionality is impaired. The mechanisms that underlie this reduced count and impaired functionality are poorly understood. Nitric oxide (NO) is a short-lived signalling molecule that is produced by vascular endothelial cells and participates in the maintenance of vascular tone. NO is also known to participate in other physiological processes, such as cell survival, proliferation, and migration. The bioavailability of NO is reduced in EPCs from DM-2 patients. Interestingly, an inverse relationship exists between the reduction in NO bioavailability in EPCs and the patient’s plasma glucose and glycated haemoglobin levels. In addition, NO bioavailability in EPCs correlates with plasma oxidized low-density lipoprotein levels in DM-2. Although this reduction in NO bioavailability could be attributed to oxidative stress in DM-2 patients, it also may be due to impairment of one or more members of the protein signalling cascades that are responsible for NO production. The stimulation of NO production or its signalling cascades in EPCs may increase their numbers and improve their function, thus attenuating endothelium damage, independent of the vasodilatory effects of NO. This review summarizes the metabolic alterations that underlie the molecular mechanisms that may be responsible for EPC decrease and dysfunction in DM-2 with emphasis on the involvement of the NO system.

Keywords

EPC • Nitric oxide • Diabetes mellitus • Hyperglycaemia • Hyperlipidaemia

1. Introduction

Diabetes mellitus type-2 (DM-2) has reached epidemic proportions worldwide and is associated with a large economic burden, an increased risk of cardiovascular disease, poor outcomes as a result of vascular occlusion, and premature mortality. Hyperglycaemia is the hallmark clinical manifestation of DM-2, and its multifactorial aetiology involves genetic, environmental, and behavioural elements. The detrimental effects of hyperglycaemia on the vasculature are exacerbated in DM-2 patients with elevated plasma lipid levels.1

Vascular endothelial function is impaired in DM-2. The clinical severity of vascular occlusive disease in DM-2 patients has, in part, been attributed to impaired collateral vessel development due to altered function of mature endothelial cells.2 There is, however, increasing evidence that neovascularization in adults may also involve bone marrow (BM)-derived endothelial progenitor cells (EPCs).3 It has been reported that the ability of DM-2 patients to develop coronary collaterals is diminished due to a diabetes-associated reduction in the EPC count and an impairment of EPC mobilization.4 Moreover, the results of several studies have demonstrated that hyperglycaemia or oxidized low-density lipoprotein (oxLDL) can reduce both the EPC count and impair EPC migration and proliferation by exerting a deleterious effect on the phosphatidylinositol-3 kinase (PI 3-K)/protein kinase B (PKB)/Akt/endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signalling cascade.5–8

Recently, we reported that concomitant hyperglycaemia and elevated oxLDL levels are associated with diabetic vasculopathy.9 We also demonstrated that the exposure of EPCs to either high glucose or oxLDL concentrations exerted a deleterious effect on the PI 3-K/Akt/eNOS signalling cascade, which was exacerbated when EPCs were simultaneously exposed to both compounds. The aim of this review is to present the putative metabolic alterations that underlie the molecular mechanisms that are responsible for decreased

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EPC count and functionality in DM-2 with special attention to the involvement of the NO system in this phenomenon.

2. Endothelial progenitor cells

In 1997, Asahara et al. demonstrated that BM-derived CD34+/vascular endothelial growth factor receptor (VEGFR)-2+ monocytes can be isolated from human blood and can be grown in culture under conditions that yield colonies of cells which are characterized by the expression of surface markers of mature endothelial cells, such as CD31, E-selectin, von Willebrand factor, and eNOS, and the uptake of fluorescent-tagged acetylated LDL. Over the years, it was found that these BM-derived cells are very important for the maintenance of endothelial integrity and function, as well as for post-natal neovascularization. EPCs can be quantified by the number of circulating CD34+/VEGFR-2+ or CD34+/VEGFR-2+/CD133+ cells or by the number of colonies of adherent cells that can be obtained from circulating mononuclear cells (MNCs) that express mature endothelial cell markers. Clinically, the number and function of EPCs may reflect the balance between endothelial integrity and repair, and both measures have been suggested as surrogate markers of endothelial function and cardiovascular diseases.

3. Isolation and identification of EPCs

The exact definitions, the origin, and identification of EPCs isolated after culturing peripheral blood (PB)-MNCs in a medium that favours endothelial differentiation are controversial because different sources for endothelial cells exist: (i) haematopoietic stem cells, (ii) myeloid cells, (iii) circulating mature endothelial cells, which may also shed off the vessel wall, and (iv) other circulating progenitor cells. Several groups have identified a rare population of highly proliferative endothelial colony-forming cells from both umbilical cord blood and adult PB-MNCs that exhibits all the properties of progenitor cells. However, the outgrowth of endothelial cells from cultures of BM-derived PB-MNCs showed more than a 1000-fold expansion compared with circulating endothelial cells that originate from vessel walls.

Two isolated types of EPCs derived from human PB have been described: early EPCs and late EPCs which have comparable angiogenic capabilities. Early EPCs are similar to the progenitor cells first reported by Asahara et al. These cells have been referred to as monocyte-derived circulating angiogenic cells expressing CD14. They have a spindle-shaped phenotype, but they do not give rise to endothelial outgrowth, whereas the CD14- cells give rise to endothelial outgrowth. Late EPCs have a cobblestone appearance and are similar to the circulating BM-derived endothelial cells that give rise to outgrowth. These two types of EPCs have different proliferation rates and survival behaviours. Although they also have different gene expression profiles, which lead to different functions in vitro, they equally contribute to neovascularogenesis in vivo: early EPCs secrete angiogenic cytokines and late EPCs supply a sufficient number of endothelial cells.

Collectively, the results of these studies suggest three general approaches for identifying EPCs: (i) isolating PB-MNCs from the blood, and then culturing them on fibronectin-coated tissue culture plates with various endothelial growth factors, (ii) utilizing monoclonal antibodies and fluorescence-activated cell sorting analysis to enumerate specific cell populations, and (iii) using in vitro colony-forming cell assays. The results of some studies suggest that no specific or unique marker can be used to define EPCs in humans and experimental animals.

4. EPCs and vascular risk factors

The results of several studies indicate that EPC count and function, including mobilization, proliferation, and attachment, inversely correlate with cardiovascular risk factors. For example, the count and migratory ability of circulating EPCs of patients with coronary artery disease (CAD) are substantially lower than those of age-matched healthy individuals or individuals with high serum LDL cholesterol levels. Hill et al. measured EPC colony numbers and endothelium-dependent vasodilatation in 45 men with varying cardiovascular risk and found a powerful positive correlation between EPC colony number and endothelium-dependent vasodilatation, and a powerful negative correlation between EPC colony number and the Framingham risk score. Lambiase et al. reported that the EPC count in patients with poor coronary collateral circulation was lower than that of healthy individuals. In addition, an increased EPC count is associated with a reduced risk of death from a first major cardiovascular event, revascularization surgery, and hospitalization. The results of these studies indicate that EPCs are important for vascular health, thus advocating research into the underlying mechanisms that are responsible for impaired EPC count and function in various vascular diseases.

5. DM-2, vascular complications, and EPCs

The aetiology of DM-2-associated vascular damage has been explored in depth, but its pathophysiology is complex, multifactorial, and not fully understood. The clinical manifestations of cardiovascular disease in DM-2 individuals are associated with poor endothelial function and a decreased ability of the endothelium to regenerate and maintain its integrity. Adults with DM-2 have a two- to four-fold increased risk of cardiovascular events compared with those without DM-2. DM-2 is directly implicated in various cardiovascular diseases that include stroke, ischaemic heart disease, and peripheral vascular disease. The vascular complications in DM-2 patients can be caused by macro-angiopathy which mainly consists of accelerated atherosclerosis that affects the coronary, carotid, and peripheral arteries, and increase the risk of myocardial infarction, stroke, and diabetic foot disease. However, the mechanisms of endothelial dysfunction induced by DM-2 include reduced bioavailability of endothelial cell-derived NO, which is an independent predictor of cardiovascular events and resistance to the non-metabolic effects of insulin, hyperglycaemia, hyperlipidaemia, and oxidative stress.

Reduced availability and down-regulation of EPCs are among several important mechanisms that are responsible for the occurrence of endothelial dysfunction and vascular diseases. Decreased number and impaired function of EPCs can be involved early in endothelial dysfunction and atherogenesis and later in impaired collateralization after artery occlusive diseases, leading to clinical manifestations of vascular diseases. Emerging evidence suggests that type 1 diabetes mellitus and DM-2 are both associated with reduced numbers and
impaired function of EPCs. Tepper et al. showed that EPCs isolated from patients with DM-2 displayed impaired proliferation, adhesion, and attachment to activated human umbilical vein endothelial cells. This group also showed that EPC proliferation from these diabetic patients was inversely correlated with their plasma glycated haemoglobin (HbA1c) levels, suggesting a relationship between the patients' glycaemic control and EPC number and proliferation. Fadini et al. reported that the EPC count in DM-2 patients with peripheral arterial disease (PAD) is substantially lower than that of healthy subjects, non-diabetic patients with vascular disease, and DM-2 patients without vascular disease. The results of the Fadini study also showed that the reduced EPC number was associated with the severity of PAD in the DM-2 patients and was inversely correlated with the patients' plasma glucose levels and the number of cardiovascular risk factors. In addition, Fadini et al. have suggested that the EPC count might be considered as a novel biomarker of peripheral atherosclerosis in DM-2. In another study, the same group demonstrated that the circulating CD34-labeled cell count is inversely correlated with a cardiovascular risk profile and can be used to identify EPCs in diabetes. Although these studies suggested severe EPC impairment in DM-2 patients with vascular diseases, they provided little insight to underlying mechanisms that led to the severe reduction in EPC numbers in these patients. However, they did propose that a combination of hyperglycaemia and adverse metabolites produced by diabetes could explain the severe reduction in circulating EPC counts in DM-2 patients that lead to accelerated endothelial dysfunction, atherogenecity, and subsequent severe vascular diseases. The contribution of clustered risk factors to EPC dysfunction is further discussed below.

6. EPCs and the PI 3-K/Akt pathway

Although the molecular mechanisms that underlie the homing and recruitment of BM-derived EPCs for the remodelling of vascular tissues remain unclear, the evidence that EPCs promote vascular repair and neovascularization is strong. Emerging data suggest that the PI 3-K/Akt pathway is vital for regulating EPC recruitment, mobilization, and proliferation. Exercise and drugs, such as hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins), erythropoietin, estrogens, and VEGF, are able to increase circulating EPC count, and proliferation and migration, and all are well-known activators of the PI 3-K/Akt protein kinase pathway. Both pharmacological inhibition of PI 3-K and the overexpression of a dominant-negative Akt construct have been shown to abolish statin- and VEGF-induced EPC proliferation and differentiation in vitro and in vivo. In addition, compounds that stimulate the PI 3-K/Akt protein kinase pathway can also activate eNOS. This association between eNOS and EPC count and activity appears to be crucial because the expression of eNOS is essential for the mobilization of stem and progenitor cells, and perturbations in the PI 3-K/Akt/eNOS/NO signalling pathway or one of its members may result in EPC dysfunction.

7. The NO system and EPCs

NO is a biologically active unstable radical that is synthesized in vascular endothelial cells by eNOS, and its bioavailability depends on the balance between its production and inactivation. EPCs participate in the neovascularization process and are mobilized from BM stem cell niches to the peripheral circulation by NO and eNOS. Endogenous NOS inhibitors, such as asymmetric dimethylarginine (ADMA), may lead to endothelial dysfunction and inhibition of angiogenesis in vivo. ADMA has also been suggested as a surrogate marker for cardiovascular events or deaths, as high circulating ADMA levels were correlated with decreased EPC mobilization, differentiation, and proliferation in patients with CAD.

Decreased NO bioavailability has been proposed as one of the determinants of vascular damage in DM-2. DM-2 patients have a lower overall systemic fraction of L-arginine that is converted to NO compared with that found in healthy individuals. In addition, uncoupling of eNOS in blood vessels of diabetic patients due to a reduction in the essential eNOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) diminishes NO bioavailability. Recent studies reported that in vitro treatment of EPCs from DM-2 patients with an NO donor drug normalized their migration. Moreover, treating wounds in diabetic mice with stromal-derived factor-1α restored EPC homing to these wounds through an NO-dependent mechanism. NO bioavailability and the in vivo reendothelialization capacity of EPCs from diabetic patients were restored by inactivating NAD(P)H oxidase. Our group recently reported that the proliferation of glucose-stressed EPCs can be restored by preserving the bioavailability of NO with superoxide dismutase (SOD), emphasizing the importance of NO and oxidative stress to EPC count and proliferation.

Whether generated from the endothelium or from EPCs, NO bioavailability in sites of active vascularization seems to be critical for EPC biology and function. Vasorelaxant prostanooids such as prostacyclin (PGI2) or its derivatives exert protective effects on endothelial cells by mechanisms that partly involve cyclic adenosine monophosphate-mediated NO formation. Indeed, it was demonstrated that PGI2 analogues such as Beraprost or Iloprost increase EPC number and migration in human and in ischaemic tissues of experimental animal models. It was suggested that by mediating its beneficial effects on angiogenesis and repairing vascular walls, PGI2 has a direct effect on EPC functions in an autocrine or paracrine manner through an NO-dependent mechanism. In this regard, NO-dependent vasoprotective agents such as prostacyclin or statins could have a significant therapeutic role in cardiovascular diseases under pathological conditions, such as diabetes where the count and migratory activity of EPCs are impaired.

8. Suggested mechanisms for EPC impairment in DM-2

8.1 Effect of hyperglycaemia

Several mechanisms may be involved in hyperglycaemia-induced reduction in the number and survival of EPCs and impairment of their proliferative and migratory capacity. Some mechanisms cause a reduction in NO bioavailability and an increased senescence by activating p38 mitogen-activated protein kinase. Hyperglycaemia induced-oxidative stress in DM-2 has also been suggested as a potential mechanism for reduced EPC count and impairment and is discussed below. Contrary to the concept of oxidative stress-induced EPC damage through the NO system, Chen et al. showed that down-regulation of eNOS expression and phosphorylation by high glucose concentrations in vitro can result in reduced early and late EPC numbers and activity by mechanisms that are not associated with oxidative stress. Despite differences in gene expression and in vitro function between early and late EPCs, it was demonstrated that
eNOS is an important target for high glucose adverse effects on EPC number and activity. Meanwhile, eNOS deactivation in diabetic EPCs resulted in excessive superoxide anion production and in reduced NO bioavailability, implying an intimate relationship between oxidative stress and EPC damage. Yet, it is still unclear whether high glucose-associated eNOS damage causes oxidative stress or if it is the high glucose-associated oxidative stress that causes eNOS deactivation. The different protocols used for EPC isolation and culture in the presence of high glucose might therefore play a significant role in determining the outcomes of EPC function in vitro.

We recently showed that an inverse relationship exists between the reduced NO bioavailability in EPCs from DM-2 patients and the patients' plasma glucose and HbA1c levels. This reduction in NO bioavailability could be attributed to enhanced oxidative stress in DM-2 patients, which is known to damage the protein signalling pathways that lead to NO production.

8.2 Effect of reactive oxygen species-induced oxidative stress

As mentioned above, EPC dysfunction in DM-2 patients is associated with oxidative stress due to excessive generation of reactive oxygen species (ROS). Recently, we showed that prolonged exposure of EPCs to high glucose concentrations in vitro increased superoxide anion production and reduced NO bioavailability. Glucose stress in EPCs can result in the generation of superoxide anions by several processes that include glucose auto-oxidation, and increased protein kinase C (PKC) and NAD(P)H oxidase activity. In addition, eNOS uncoupling due to BH4 deficiency and/or to increased PKC activity leads to excessive superoxide anion production and reduced NO bioavailability and impairs EPC number and function in diabetes. Indeed, increased generation and inadequate removal of superoxide anions that result in oxidative stress may constitute a significant cause for impaired NO-mediated EPC dysfunction. This could be demonstrated by the inhibition of NAD(P)H oxidase activity in EPCs from DM-2 patients that restored their NO bioavailability and function.

Reduced extracellular SOD activity has been shown to be closely associated with increased vascular oxidative stress and has been implicated in the endothelial dysfunction of patients with hypertension, congestive heart failure, and CAD. Ceradini et al. demonstrated that the prevention of hyperglycaemia-induced ROS generation significantly improved EPC-induced revascularization in ischaemic tissues in genetically engineered diabetic mice that overexpressed SOD, or after treating diabetic mice with SOD. Neutralization of the p66ShcA gene, which regulates the apoptotic response to oxidative stress, prevented high glucose-induced EPC impairment in vitro. Human EPCs have high intracellular expression levels of manganese SOD, which plays a crucial role in protecting these cells against oxidative stress. However, it should be argued that if the increased SOD activity of EPCs from DM-2 patients is sufficient to neutralize the high levels of superoxide anion that are observed in these patients. Ohshima et al. demonstrated that antioxidant therapy with SOD in diabetic mice reduced oxidative stress and increased EPC number and potential to differentiate into endothelial cells.

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Figure 1 (A) Schematic representation of the role of ROS-mediated mechanisms in hyperglycaemia-induced EPC dysfunction. ROS are generated in hyperglycaemia. The interaction between ROS and NO produces peroxynitrite which together with high levels of LDL produces high levels of oxLDL, which together with free ROS induce severe impairment of the count and function of EPCs. (B) Schematic representation of combined hyperlipidaemia- and hyperglycaemia-induced EPC dysfunction. Hyperlipidaemia or hyperglycaemia impairs the count and function of EPCs, whereas together they aggravate this impairment. More ROS are generated in the presence of both hyperlipidaemia and hyperglycaemia. The interaction between high ROS levels and NO produces high levels of oxLDL, which together with free ROS induce severe impairment of the count and function of EPCs. ADMA, asymmetric dimethylarginine; eNOS, endothelial NO synthase; LDL, low-density lipoproteins; oxLDL, oxidized LDL; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase. injury; interaction; – – –, partial effect.
neutralization of superoxide anions. Therefore, it is possible that the addition of SOD, which catalyses the dismutation of superoxide into oxygen and hydrogen peroxide, prevented the formation of peroxynitrite, thereby increasing NO bioavailability in EPCs (Figure 1A). Collectively, our data and that of others revealed another significant mechanism that could account for the reduction of NO bioavailability in EPCs, in addition to the already known mechanism of down-regulation of eNOS expression and activation by high glucose concentrations.7

8.3 Effect of clustered risk factors
Hyperglycaemia alone seems to be insufficient to cause severe vascular complications in DM-2 patients. For example, hyperglycaemia alone was shown not to be sufficient for stimulating macrophage proliferation in atherosclerotic lesions.66 As mentioned above, Fadini et al.32–34 suggested that a combination of hyperglycaemia and adverse diabetes metabolites, such as hyperlipidaemia and advanced glycation end-products, could most likely explain the severe reduction in circulating EPC counts in DM-2 patients, which lead to accelerated endothelial dysfunction, atherogenicity, and subsequent severe vascular diseases. Vascular disease in DM-2, therefore, appears to be related to hyperglycaemia with a cluster of risk factors that include hypertension, smoking, hypercholesterolaemia, dyslipidaemia, and obesity.67

High serum levels and abnormalities of lipids that include triglycerides and LDL are associated with an increased risk of CAD in DM-2 patients.68 Hyperlipidaemia in apoE-deficient mice caused a low circulating EPC count, which correlated with enhanced atherosclerosis,69 whereas lipoprotein apheresis treatment of patients with refractory hyperlipidaemia stimulated EPC proliferation and increased eNOS activity.70

Several in vitro studies on EPCs from DM-2 patients revealed that oxLDL reduced EPC survival, count, and function, as well as their eNOS activity and NO bioavailability.8,71 We recently showed that DM-2 patients with CAD have high plasma oxLDL levels, which were inversely correlated with EPC migration and NO production.9 Thus, elevated oxLDL levels exacerbate hyperglycaemia-impaired EPC migration. We also found that EPC migration and NO production were profoundly impaired in DM-2 patients with CAD compared with EPC migration and NO production in healthy individuals. DM-2 patients without CAD, and CAD patients without DM-2.7 In the light of these findings, we proposed that the combination of hyperglycaemia and elevated plasma oxLDL levels accounts for the low EPC count and impaired EPC migration in these patients by involving the Akt/eNOS signalling pathway (Figure 1B). However, it should be noted that concomitant elevated circulating glucose and oxLDL levels are not usually found in well-controlled DM-2 patients with or without CAD, but can be seen in some uncontrolled DM-2 patients after consuming meals that are rich in carbohydrates and unsaturated fat. Concomitant elevated circulating glucose and oxLDL levels can also be seen in some stress conditions, such as in inflammation or infection, and during hospitalization. Thus, the results from our study may only be relevant for some uncontrolled DM-2 patients with CAD.

9. Conclusions
Although still not well assessed, EPC number and function could be suggested as a surrogate marker for vascular endothelial function. In DM-2 patients, the use of antioxidants and/or other medications, such as prostacyclin or statins, can enhance EPC number and function at least through NO-dependent mechanisms.

Hyperglycaemia-induced increased oxidative stress plays an important role in EPC dysfunction in DM-2. The combination of elevated plasma/serum oxLDL levels and hyperglycaemia that may be seen in some uncontrolled DM-2 patients further aggravates the impaired EPC migration and NO production observed in hyperglycaemia alone. We and others suggest that the mechanisms that are responsible for the reduced number and impaired function of EPCs in DM-2 are partially linked to the PI 3-K/Akt/eNOS/NO signalling pathway. Therefore, we suggest that either this pathway or the interaction between hyperglycaemia and hyperlipidaemia in DM-2 patients, who have vascular diseases, are potential therapeutic targets for abolishing the impaired function of EPCs and for restoring their neovascularization capacity.

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