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Endothelial Progenitor Cell Dysfunction in Diabetes Mellitus Type-2: Focus on Nitric Oxide System

Saher Hamed
Department of Cardiology, Rambam Health Care Campus, Haifa & Cardiovascular Research Laboratory, Rappaport Faculty of Medicine, Technion Haifa, Israel

1. Introduction

Diabetes mellitus type-2 (DM-2) is a global epidemic that is associated with a large economic burden, an increased risk of cardiovascular disease, poor outcomes as a result of vascular occlusion, and premature mortality. The clinical hallmark of DM-2 is hyperglycemia – with an etiology which involves genetic, environmental, and behavioral elements. Vascular endothelial function is impaired in DM-2. The underlying cause for the clinical severity of vascular occlusive disease in DM-2 patients has been partly attributed to impaired collateral vessel development due to altered function of mature endothelial cells (Abaci et al., 1999). Furthermore, increasing evidence suggest that new blood-vessel formation in adults may also involve bone marrow (BM)-derived endothelial progenitor cells (EPCs) (Asahara et al., 1997). The ability of DM-2 patients to develop coronary collaterals is diminished due to a diabetes-associated reduction in EPC count and an impairment of EPC mobilization (Lambiase et al., 2004). The deleterious effects of hyperglycemia on the vasculature are further exacerbated in DM-2 patients with elevated plasma lipid levels (Kanter et al., 2007). Several studies have shown that hyperglycemia or oxidized low-density lipoprotein (oxLDL) can reduce EPC count as well as impair EPC migration and proliferation by exerting harmful effects on the phosphatidylinositol-3 kinase (PI 3-K)/protein kinase B (PKB/Akt)/endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signaling cascade (Callaghan et al., 2005; Chen et al., 2007; Krankel et al., 2005; Ma et al., 2006).
This review presents and discusses the underlying metabolic alterations to the molecular mechanisms that are responsible for decreased EPC count and functionality in DM-2. The involvement of the NO system in this phenomenon is highlighted.

2. Endothelial Progenitor Cells (EPCs)

In 1997, BM-derived $CD34^+$/vascular endothelial growth factor receptor (VEGFR)-2^+ monocytes were first isolated from human blood by Asahara and co-workers and grown in culture under conditions that yielded colonies of cells which were characterized by the expression of surface markers of mature endothelial cells - CD31, E-selectin, von Willebrand factor (vWF), eNOS - and by the uptake of fluorescent-tagged acetylated LDL (Asahara et

al., 1997). It was later discovered that these BM-derived cells are very important for the maintenance of endothelial integrity and function, as well as for postnatal new blood vessel formation (Rafii & Lyden, 2003). EPCs can be quantified by the number of circulating $CD34^+/VEGFR\text{-}2^+$ or $CD34^+/VEGFR\text{-}2^+/CD133^+$ 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 (Peichev et al., 2000). The number of EPCs and their functionality level can serve as surrogate markers of endothelial function and cardiovascular diseases because these measures are indicative of the balance between endothelial integrity and repair.

2.1 Isolation and identification of EPCs

There are different sources for endothelial cells: hematopoietic stem cells, myeloid cells, circulating mature endothelial cells (which may also shed off the vessel wall), and other circulating progenitor cells. Therefore, there are no exact definitions for the origin and identification of EPCs isolated after culturing peripheral blood (PB)-MNCs in a medium that favors endothelial differentiation (Urbich & Dimmeler, 2004). A rare population of **highly proliferative endothelial colony-forming cells** from umbilical cord blood and from adult PB-MNCs that exhibit all the properties of progenitor cells **were also identified (**Ingram et al., 2004; Yoder et al., 2007). Furthermore, the outgrowth of endothelial cells from cultures of BM-derived PB-MNCs had an expansion rate that was more than a 1000-fold of that of circulating endothelial cells originating 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 but different proliferation rates and survival behaviors (Hur et al., 2004). Early EPCs have a spindle-shaped phenotype but they do not give rise to endothelial outgrowth. These cells have also been referred to as monocyte-derived circulating angiogenic cells expressing CD14 (Gulati et al., 2003). Late EPCs have a cobblestone appearance and are similar to the circulating BM-derived cells that give rise to endothelial outgrowth (Gulati et al., 2003; Lin et al., 2004). Although they also have different gene expression profiles, which lead to different functions *in vitro*, they equally contribute to new blood vessel formation *in vivo*: early EPCs secrete angiogenic cytokines, and late EPCs supply a sufficient number of endothelial cells (Hur et al., 2004).

Three general approaches for identifying EPCs have been suggested by these studies: (1) isolating PB-MNCs from the blood, and then culturing them on fibronectin-coated tissue culture plates with various endothelial growth factors, (2) utilizing monoclonal antibodies and fluorescence-activated cell sorting (FACS) analysis to enumerate specific cell populations, and (3) using *in vitro* colon-forming cell assays. Some studies suggested that no specific or unique marker can be used to define EPCs in humans and experimental animals (Griese et al., 2003; Kocher et al., 2001; Timmermans et al., 2009).

2.2 EPCs and vascular risk factors

EPC count and function - mobilization, proliferation and attachment - inversely correlate with cardiovascular risk factors. This is demonstrated by the finding that 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 (Vasa et al., 2001). A powerful positive correlation between EPC colony number and endothelium-dependent vasodilatation was found in 45 men with

varying cardiovascular risk, while a powerful negative correlation was found in these men between EPC colony number and the Framingham risk score (Hill et al., 2003). The EPC count in patients with poor coronary collateral circulation was reported to be lower than that of healthy individuals (Lambiase et al., 2004). In addition, an increased EPC count is associated with a reduced risk of death from a first major cardiovascular event, revascularization surgery, and hospitalization (Werner et al., 2005). 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.

2.3 DM-2, vascular complications and EPCs

The pathophysiology of DM-2-associated vascular damage is complex, multi-factorial, and not fully understood. A two- to fourfold increased risk of cardiovascular events exists in adults with DM-2 compared with those without DM-2 (Fox et al., 2004) and DM-2 is directly implicated in various cardiovascular diseases that include stroke, ischemic 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 (Goldberg, 2003).

Cardiovascular disease in individuals with DM-2 is associated with endothelial dysfunction which is manifested by reduced bioavailability of endothelial cell-derived NO, resistance to the non-metabolic effects of insulin, hyperglycemia, hyperlipidemia, and oxidative stress (Anderson, 2003; Griendling et al., 2003), and with a decreased ability of the endothelium to regenerate and maintain its integrity (McVeigh et al., 1992). One of the important mechanisms for endothelial dysfunction and vascular diseases is reduced availability and downregulation of EPCs (Quyyumi, 2004). 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 (Landmesser et al., 2004).

Both type 1 diabetes mellitus and DM-2 are associated with reduced numbers and impaired function of EPCs; EPCs isolated from patients with DM-2 displayed impaired proliferation, adhesion, and attachment to activated human umbilical vein endothelial cells and the proliferation of EPCs the same diabetic patients was inversely correlated with their plasma glycated hemoglobin (HbA1c) levels, suggesting a relationship between the patients' glycemic control and EPC number and proliferation (Tepper et al., 2002). The EPC count in DM-2 patients with peripheral arterial disease (PAD) was shown to be substantially lower than that of healthy subjects, non-diabetic patients with vascular disease, and DM-2 patients without vascular disease (Fadini et al., 2005). The same 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. These findings led to the suggestion that EPC count could serve as a biomarker of peripheral atherosclerosis in DM-2 (Fadini et al., 2006). In another study, the same group demonstrated that the circulating $CD34^+$ cell count is inversely correlated with a cardiovascular risk profile and can be used to identify EPCs in diabetes (Fadini et al., 2006a). Although these studies demonstrated the existence of severe EPC impairment DM-2 patients with vascular diseases, they did not provide much information regarding the mechanisms that lead to the severe reduction in EPC numbers in diabetic patients. It was

suggested, however, that the severe reduction in circulating EPC counts may be caused by a combination of hyperglycemia and adverse metabolites produced by diabetes which in turn could lead to accelerated endothelial dysfunction, atherogenecity, and subsequent severe vascular diseases. The contribution of clustered risk factors to EPC dysfunction is further discussed below.

2.4 EPCs and the PI 3-K/Akt pathway

The molecular mechanisms that underlie the homing and recruitment of BM-derived EPCs for the remodeling of vascular tissues remain unclear, but there is strong evidence that EPCs promote vascular repair and new blood vessel formation. EPC recruitment, mobilization and proliferation are regulated by the PI 3-K/Akt pathway (Morello et al., 2009). Exercise and drugs, such as hydroxymethylglutaryl-coenzyme A reductase inhibitors (statins), erythropoietin, estrogens, and vascular endothelial growth factor (VEGF), are known activators of the PI 3-K/Akt protein kinase pathway which increase circulating EPC count, proliferation and migration (Bahlmann et al., 2004; Dimmeler et al., 2001). Statin- and VEGF-induced EPC proliferation and differentiation were abolished *in vitro* and *in vivo* by pharmacological inhibition of PI 3-K as well as by the overexpression of a dominant negative Akt construct (Dimmeler et al., 2001). Furthermore, since compounds that stimulate the PI 3-K/Akt protein kinase pathway can also activate eNOS (Fulton et al., 1999), an association between eNOS and EPC count and activity appears to be essential because the expression of eNOS is necessary for the mobilization of stem and progenitor cells (Aicher et al., 2003), and disturbances in the PI 3-K/Akt/eNOS/NO signaling pathway or one of its members may result in EPC dysfunction.

2.5 The NO system and EPCs

EPCs participate in formation of new blood vessels. They are mobilized from BM stem cell niches to the peripheral circulation by NO and eNOS (Aicher et al., 2003; Ozuyaman et al., 2005). Nitric oxide 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 (Wattanapitayakul et al., 2000). Asymmetric dimethylarginine (ADMA) – an endogenous NOS inhibitor - may lead to endothelial dysfunction and inhibition of angiogenesis *in vivo* (Boger & Bode-Boger 2000), and it has 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 (Thum et al., 2005).

One of the determinants of vascular damage in DM-2 is decreased NO bioavailability. DM-2 patients have a lower overall systemic fraction of L-arginine that is converted to NO compared with that found in healthy individuals (Avogaro et al., 2003). An additional factor that leads to diminished NO bioavailability in blood vessels of DM-2 patients is the reduction in the essential eNOS cofactor - (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4) - which leads to the uncoupling of eNOS in blood vessels (Bauersachs & Schäfer , 2005). The migration of EPCs taken from DM-2 patients was normalized after treatment with an NO donor drug (Segal et al., 2006). Moreover, an NO-dependent mechanism was responsible for restoring EPC homing in diabetic wounds in mice which were treated with stromal derived factor-1 alpha (SDF1α), (Gallagher et al., 2007). Inactivation of NAD(P)H oxidase restored NO bioavailability and the *in vivo* re-endothelialization capacity of EPCs from diabetic

patients (Sorrentino et al., 2007). 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 (no self citation is allowed..).

NO bioavailability in sites of active vascularization seems to be critical for EPC biology and function. Vasorelaxant prostanoids such as prostacyclin (PGI_2) or its derivatives exert protective effects on endothelial cells by mechanisms that partly involve cyclic adenosine monophosphate (cAMP)-mediated NO formation (Niwano et al., 2003). Indeed, it was demonstrated that PGI_2 analogues such as Beraprost or Iloprost increase EPC number and migration in human and in ischemic tissues of experimental animal models (di Stefano et al., 2008; Miyahara et al., 2006). It was suggested that by mediating its beneficial effects on angiogenesis and repairing vascular walls, PGI_2 has a direct effect on EPC functions in an autocrine or paracrine manner through an NO dependent mechanism (Kawabe et al., 2010). 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.

3. Suggested mechanisms for EPC impairment in DM-2

3.1 Effect of hyperglycemia

Hyperglycemia induces a reduction of the number of EPCs, their survival ability and impairs their proliferative and migratory capacity. Several mechanisms are involved in this process: some mechanisms cause a reduction in NO bioavailability (Krankel et al., 2005) and an deterioration by activating p38 mitogen-activated protein kinase (Kuki et al., 2006). Oxidative stress induced by hyperglycemia has also been suggested as a potential mechanism for reduced EPC count and impairment (Callaghen et al., 2005) and is discussed below. Contrary to the concept of oxidative stress-induced EPC damage through the NO system, *in vitro* down-regulation of eNOS expression and phosphorylation by high glucose concentrations resulted in reduced numbers and activity of early and late EPCs through mechanisms that were not associated with oxidative stress (Chen et al., 2007). Despite differences in gene expression and *in vitro* function between early and late EPCs (Hur et al., 2004), it was demonstrated that eNOS is an important target for high glucose adverse effects on EPC number and activity. While, eNOS deactivation in diabetic EPCs resulted in excessive superoxide anion production and in reduced NO bioavailability (Thum et al., 2007), implying an intimate relationship between oxidative stress and EPC damage, it is still unclear if 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*.

Enhanced oxidative stress in DM-2 patients was shown to damage the protein signaling pathways that lead to NO production (Cohen & Tong 2010).

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 signaling pathways that lead to NO production (Cohen & Tong, 2010).

3.2 Effect of reactive oxygen species-induced oxidative stress

EPC dysfunction in diabetic patients can be also caused by excessive generation of reactive oxygen species (ROS) also leads to (Galasso et al., 2006). We showed that prolonged exposure of EPCs to high glucose concentrations *in vitro* increased superoxide anion production and reduced NO bioavailability. Several processes that are related to glucose stress in EPCs take place: glucose auto-oxidation, increased protein kinase C (PKC), and NAD(P)H oxidase activity lead to generation of superoxide anions. In addition, eNOS uncoupling due to BH4 deficiency or/and to increased PKC activity also lead to excessive superoxide anion production and to reduced NO bioavailability. All these processes impair EPC number and function (Thum et al., 2007). This was demonstrated by the inhibition of NAD(P)H oxidase activity in EPCs from DM-2 patients that restored their NO bioavailability and function (Sorrentino et al., 2007).

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 (Giansante & Fiotti, 2006), congestive heart failure, and CAD (Landmesser et al., 2000). Ceradini *et al.* demonstrated that prevention of hyperglycemia-induced ROS generation significantly improved EPC-induced revascularization in ischemic tissues in genetically-engineered diabetic mice that overexpressed SOD, or after treating diabetic mice with SOD (Ceradini et al., 2008). Neutralization of the $p66^{ShcA}$ gene, which regulates the apoptotic response to oxidative stress, prevented high glucose-induced EPC impairment *in vitro* (di Stefano et al., 2008). Human EPCs have high intracellular expression levels of manganese SOD, which plays a crucial role in protecting these cells against oxidative stress (Dernbach et al., 2004; He et al., 2004). 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 count and potential to differentiate into endothelial cells (Ohshima et al., 2009). We reported that treating glucose-stressed EPCs with SOD restored their proliferative ability through an NO-dependent mechanism and we suggested that the extent of the interaction between NO and superoxide anion is important to the development of EPC dysfunction since the resultant product, peroxynitrite, can reduce the EPC count and impair their proliferation. This could provide a possible mechanism for the development of cardiovascular disease in patients with DM-2. Tao and colleagues demonstrated that augmentation of SOD expression in human EPCs by shear stress can accelerate the neutralization of superoxide anions (Tao et al., 2007). Therefore, it is possible that the addition of SOD, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, prevented the formation of peroxynitrite, thereby increasing NO bioavailability in EPCs (Figure 1). Our data and that of others revealed a significant mechanism that could account for the reduction of NO bioavailability in EPCs, in addition to the already known mechanism of downregulation of eNOS expression and activation by high glucose concentrations (Callaghan et al., 2005).

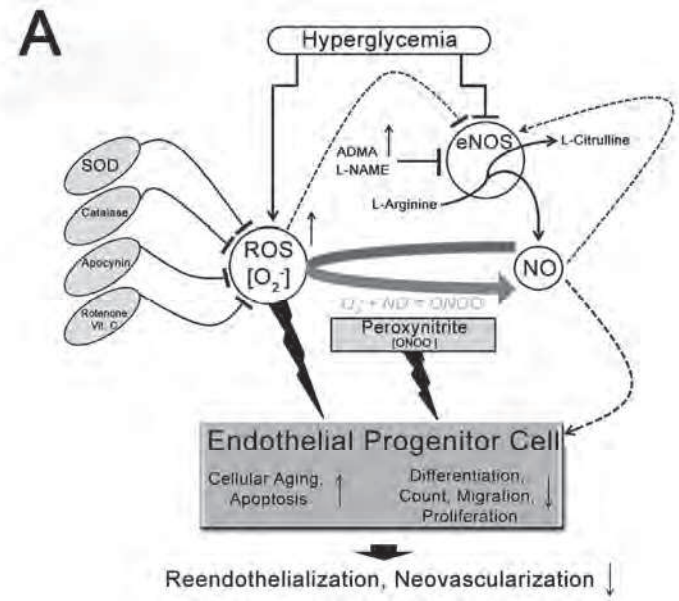

Fig. 1. Schematic representation of the role of ROS-mediated mechanisms in hyperglycemia-induced EPC dysfunction. ROS are generated in hyperglycemia. The interaction between ROS and NO produces peroxynitrite which together with free ROS impairs the count and function of EPCs. ADMA, asymmetric dimethyl-arginine; 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.

3.3 Effect of clustered risk factors

Hyperglycemia alone seems to be insufficient to cause severe vascular complications such as stimulating macrophage proliferation in atherosclerotic lesions (Lamharzi et al., 2004) in DM-2 patients. Rather, a combination of hyperglycemia and adverse diabetes metabolites, such as hyperlipidemia and advanced glycation end products, could most likely explain the severe reduction in circulating EPC counts in DM-2 patients, that lead to accelerated endothelial dysfunction, atherogenecity, and subsequent severe vascular diseases (Fadini et al., 2005; 2006; 2006a). Vascular disease in DM-2, therefore, appears to be related to hyperglycemia with a cluster of risk factors that include hypertension, smoking, hypercholesterolemia, dyslipidemia, and obesity (Caballero, 2003).

High serum levels and abnormalities of lipids that include triglycerides and LDL are associated with an increased risk of CAD in DM-2 patients (Shimada et al., 2004). Hyperlipidemia in apoE-deficient mice caused a low circulating EPC count, which correlated with enhanced atherosclerosis (Xu et al., 2003), while lipid aphaeresis treatment of patients with refractory hyperlipidemia stimulated EPC proliferation and increased eNOS activity (Patschan et al., 2009).

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

(Imanishi et al., 2004; Ma et al., 2006). Elevated oxLDL levels exacerbate hyperglycemia-impaired EPC migration; we recently showed that DM-2 patients with CAD have high plasma oxLDL levels, which were inversely correlated with EPC migration and NO production. 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. These findings led us to propose that the combination of hyperglycemia and elevated plasma oxLDL levels account for the low EPC count and impaired EPC migration in these patients by involving the Akt/eNOS signaling pathway (Figure 2). The results from our study may only be relevant for some uncontrolled DM-2 patients with CAD because 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 are also observed in some stress conditions, such as in inflammation or infection, and during hospitalization.

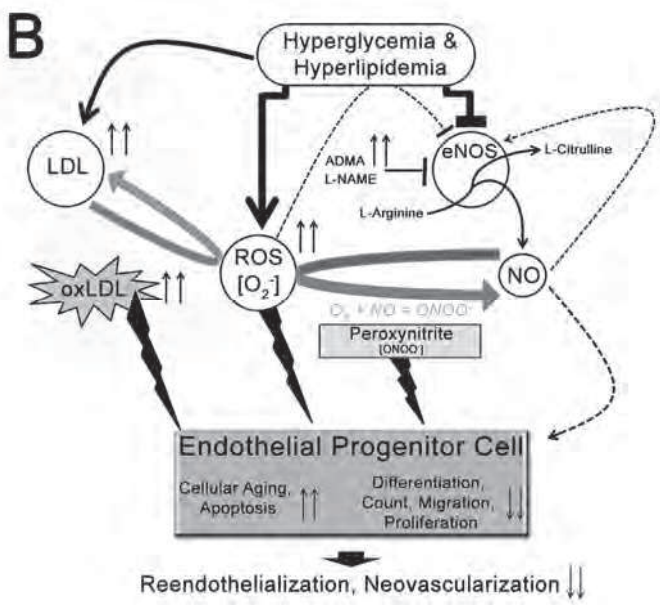

Fig. 2. Schematic representation of combined hyperlipidemia and hyperglycemia-induced EPC dysfunction. Hyperlipidemia or hyperglycemia impairs the count and function of EPCs, while together they aggravate this impairment. More ROS are generated in the presence of both hyperlipidemia and hyperglycemia. The interaction between high ROS levels and NO produces high peroxynitrite levels, and the interaction between high ROS and high levels of LDL produces high levels of oxLDL, which together with free ROS induce severe impairment of the count and function of EPCs. ADMA, asymmetric dimethyl-arginine; 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.

4. Conclusions

Hyperglycemia-induced oxidative stress plays an important role in EPC dysfunction in DM-2. The combination of elevated plasma/serum oxLDL levels and hyperglycemia that may be seen in some uncontrolled DM-2 patients further aggravates the impaired EPC migration and NO production observed in hyperglycemia alone. 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 signaling pathway. Therefore, we suggest that either this pathway or the interaction between hyperglycemia and hyperlipidemia in DM-2 patients, who have vascular diseases, are potential therapeutic targets for abolishing the impaired function of EPCs. The use of antioxidants and/or other medications, such as prostacyclin or statins, can enhance EPC number and function and restore their capacity for forming new blood vessels, at least through NO-dependent mechanisms.

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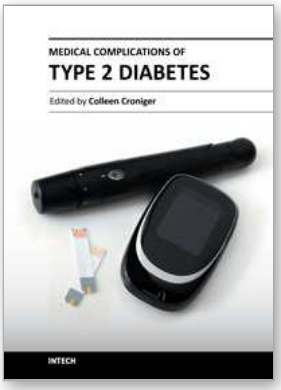

Medical Complications of Type 2 Diabetes

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Obesity and type 2 diabetes are increasing worldwide problems. In this book we reviewed insulin secretion in both healthy individuals and in patients with type 2 diabetes. Because of the risk associated with progression from insulin resistance to diabetes and cardiovascular complications increases along a continuum, we included several chapters on the damage of endothelial cells in type 2 diabetes and genetic influences on endothelial cell dysfunction. Cardiovascular complications occur at a much lower glucose levels, thus a review on the oral glucose tolerance test compared to other methods was included. The medical conditions associated with type 2 diabetes such as pancreatic cancer, sarcopenia and sleep disordered breathing with diabetes were also discussed. The book concludes with several chapters on the treatments for this disease offering us hope in prevention and successful alleviation of the co-morbidities associated with obesity and type 2 diabetes.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821