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Forum Review

Hyperglycemia-Induced Reactive Oxygen Species and Impaired Endothelial Progenitor Cell Function

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ABSTRACT

Vascular complications in diabetes are a significant source of human morbidity and mortality, affecting multiple organ systems and persisting despite tight glucose control. Many of these complications can be linked to impairments in vasculogenesis, the process by which circulating and bone marrow-derived endothelial progenitor cells (EPCs) contribute to new vessel formation. Recent evidence suggests that hyperglycemia alone, through the mitochondrial overproduction of reactive oxygen species (ROS), can induce changes in gene expression and cellular behavior in diabetes. In this review, we examine how hyperglycemia-induced overproduction of ROS could explain EPC impairments observed in diabetes. Experimentally, impairments in EPC function prevent new blood vessel growth and are potentially reversible by manipulations to decrease ROS. Novel strategies aimed at reducing hyperglycemia-induced ROS may be a useful adjuvant to antihyperglycemic therapies in the restoration of vasculogenesis and the prevention of diabetic complications. *Antioxid. Redox Signal.* 7, 1476–1482.

INTRODUCTION

DIABETES ranks as one of the leading disorders worldwide, with prevalence expected to grow by 6–10% this decade, reaching an estimated 300 million patients by 2010 (41). The majority (90–95%) of these are type II diabetes, characterized by hyperglycemia despite normal or high insulin levels (4). The consequences of type II diabetes affect metabolic, neurological, cardiovascular, and microvascular function. Vascular complications are protean, and can manifest as aberrant vascular growth (*e.g.,* retinopathy, glomerular nephropathy), accelerated atherosclerosis (1), peripheral vascular disease, cerebrovascular disease (33), and wound-healing problems. Underlying microvascular dysfunction and abnormalities in new blood vessel growth have been associated with these complications (33, 52). Under normal conditions, primary vessel loss after an ischemic insult is compensated by development of new vessels and the expansion of secondary microvascular networks, which are termed collaterals. In diabetes, microcirculatory beds do not expand or regenerate sufficiently after ischemic or injury-induced damage, preventing full restoration of blood flow and resulting in organ-specific vasculopathies (52).

Collateral vessel formation in adults has been shown to occur through angiogenesis and, more recently, vasculogenesis (Fig. 1) (6). Angiogenesis describes the formation of capillaries from preexisting vessels, based on endothelial spouting or microvascular growth (8) and is widely accepted to contribute to adult neovascularization after ischemic insult and injury (47). Abnormal angiogenesis in diabetes has been linked to retinopathy, nephropathy, poor wound healing, and transplant intolerance (33). Vasculogenesis is neovascularization that occurs through differentiation of circulating cells. Until recently, this was considered to occur only during embryogenesis, when the vasculature initially forms *in situ* (40). However, numerous studies have shown that neovascularization in adults occurs through the action of circulating bone marrow-derived cells called endothelial progenitor cells (EPCs) (6).

The isolation of these cells over a decade ago was critical to confirming the existence of postnatal vasculogenesis.

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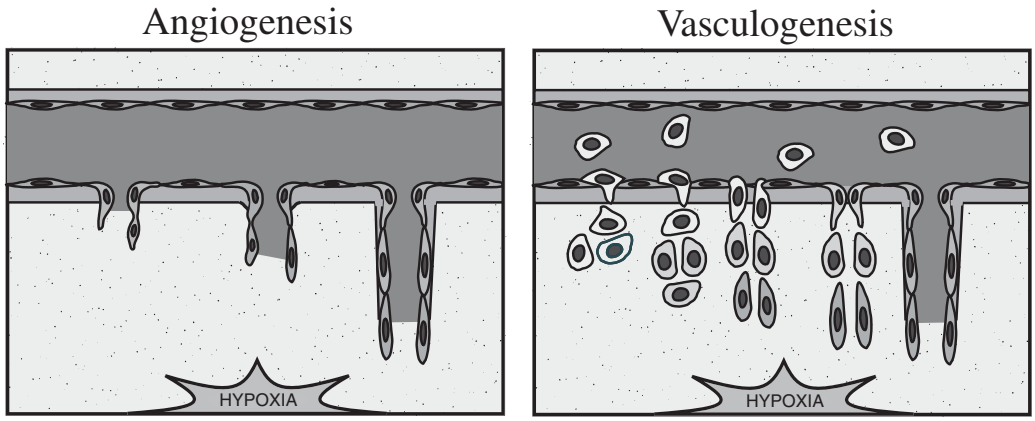

FIG. 1. Adult blood vessel formation in response to hypoxia. (**Left panel**) Angiogenesis: sprouting of vessels from existing endothelium. (**Right panel**) Vasculogenesis: development of new vessels from circulating EPCs.

EPCs derived from bone marrow are believed to be similar to embryonic angioblasts, which are mesoderm-derived cells that differentiate and proliferate to form the early vasculature. Both EPCs and angioblasts proliferate and migrate in response to stimuli such as ischemia or injury. During embryogenesis, as blood islands form in the yolk sac, the vascular progenitors are present on the periphery and blood-forming cells occupy the center (39).

In adults, the majority of EPCs reside in the bone marrow, with a smaller fraction circulating in peripheral blood and adult tissue (11, 31, 49). Although the exact characterization of adult EPCs remains an area of intense debate (5), the markers proposed include Flk-1, c-Kit, Sca-1, CD34, and AC133 (37). Hypoxia and trauma are thought to induce cytokine production (*e.g.,* vascular endothelial growth factor (VEGF), granulocyte colony stimulating factor, stromal cell-derived factor-1), which stimulates EPC mobilization, with subsequent homing and differentiation into mature endothelium at the affected site (12, 20, 53). Although the precise mechanism through which EPCs mobilize, migrate, and differentiate remains an area of intense investigation (6, 9, 38, 53), this process has been demonstrated in peripheral vascular disease, myocardial ischemia, stroke, wound healing, and tumor growth (7, 13, 17, 26).

In diabetes, EPCs have been shown by our laboratory and others to be functionally defective with respect to their ability to mobilize, migrate, and differentiate/incorporate into existing vasculature (52). This impairs ischemia-induced neovascularization and may result in an increase in ischemic complications. Early studies to treat diabetic complications have targeted EPC dysfunction by using either bone-marrow transplants from nondiabetic donors (15) or supraphysiologic injections of healthy EPCs (45) with some success. Although not reversing the inherent defects in diabetic EPCs, the dramatic effect on neovascularization seen in these studies reveals the extent to which vasculogenesis and collateral formation are dependent on EPC function. More recent studies have sought to expand the EPC population *ex vivo* and use genetic modification to overexpress angiogenic growth factors. This is followed by reintroduction of the expanded, modified colonies into circulation (28). However, with the controversy surrounding human gene therapy and the fact that underlying impairments in cell function are not addressed (52), clinical translation of these studies has been slow.

Thus, despite the high mortality and morbidity associated with the vascular complications of diabetes, current treatment is focused primarily on glucose handling through insulin treatment and oral hypoglycemic therapy. Although effective in treating many diabetic complications, this approach is limited by high costs, the life-long duration of treatment required, and the failure of many patients to reach or maintain adequate glucose levels (41). Recent evidence (described below) suggests that the microvascular complications of diabetes are caused primarily by hyperglycemia, which initiates a process of superoxide free radical overproduction through changes in the mitochondrial electron-transport chain (10). Thus, targeting oxidative stress management to restore the vasculogenic response may be a novel approach for the prevention and treatment of diabetic complications.

OXIDATIVE STRESS IN DIABETES

Free radicals are highly reactive molecules and include reactive oxygen species (ROS) (superoxide and the hydroxyl radical), reactive nitrogen species, and reactive chloride species. It is the ROS that pose the greatest threat in diabetes (36).

Under normal conditions, oxygen free radicals play a substantial role in immune defense in neutrophils and macrophages and are continually produced as by-products of the mitochondrial respiratory chain. Mitochondria are equipped with multiple defense mechanisms to convert the majority of superoxide radicals to less harmful compounds. Most potent are the antioxidant enzyme manganese superoxide dismutase (MnSOD), which catalyzes superoxide to hydrogen peroxide, and the mitochondrial isoforms glutathione peroxide and peroxiredoxin III, which detoxify hydrogen peroxide. Replenishment of the mitochondrial pool of these enzymes is dependent on sufficient levels of mitochondrial NADPH. Less active defenses include mitochondrial vitamin E and coenzyme Q (CoQ), which help prevent lipid peroxidation (23).

It is well known that diabetes is accompanied by an increase in oxidative stress (21), producing free radicals and ROS in excess of the amount of antioxidant defense. However, only recently has a mechanism linking ROS overproduction to hyperglycemia been proposed (10). The work of Michael Brownlee's lab has demonstrated that as the circulating glucose load is increased, Krebs-cycle activity increases and electron donors are overproduced in the electron-transport chain. This excess generates a higher proton threshold gradient across the mitochondrial membrane, eventually inhibiting electron transfer from CoQ to complex III (Fig. 2). Electrons transferred to CoQ are instead released within the mitochondria and used to generate superoxide radicals from molecular oxygen. As ROS production is increased, four biochemical pathways known to induce significant cellular damage in diabetes are activated: (a) increased polyol flux, (b) increased intracellular advanced glycosylation end products (AGE), (c) activation of protein kinase C, and (d) increased hexosamine pathway flux (10) (Fig. 3). In mature endothelial cells, these changes are manifested by increased apoptosis, increased cell–cell adhesion, and decreased angiogenic potential, all of which induce atherogenesis and coagulation (51). The binding of AGE to RAGE (receptor for AGE) activates the redox-sensitive transcription factors nuclear factor-κB (46), which promotes expression of the vascular cell adhesion molecule-1 and conversion to a procoagulative state (57). ROS overproduction has also been shown to inhibit the up-regulation of VEGF, an angiogenic growth factor required for neovascularization and known to be deficient in diabetes (19).

In EPCs, antioxidant enzyme expression is known to be enhanced (14), perhaps to increase their survival within the oxygen-poor environment of the bone marrow and to augment the ability of EPCs to engraft within ischemic tissue during vasculogenesis (12). Even so, it has been demonstrated that when high levels of oxidative stress are present, cell proliferation and migration are significantly impaired (14). These impairments were similar to the baseline proliferation and migration deficiencies seen in diabetic EPCs (52). Therefore, it is conceivable that the biochemical basis for EPC dysfunction in diabetes is excessive oxidative stress in the form of oxygen free radicals, similar to that described above in mature endothelial cells (14, 19, 57). This would account for both inherent EPC dysfunction in diabetic patients (52) and *in vivo* functional deficits seen during ischemia and injury-induced vasculogenesis in diabetes (19, 52). If this is the case, treatment aimed at reversing oxidative stress in EPCs may represent a powerful alternative to current diabetic vascular therapy.

THERAPIES

There is very little information regarding the dysfunction of antioxidant defenses in progenitor cell subsets derived from diabetic patients, despite significant evidence in mature cell types. This is currently an investigative focus of our laboratory. The establishment of oxidative stress as a mediator of diabetic vascular damage raises the question of whether therapies aimed at mitochondrial and antioxidant processes might have an additive effect on the prevention of diabetic complications, especially as improved glycemic control does not reduce oxidative stress in diabetics (56). Treatments proposed

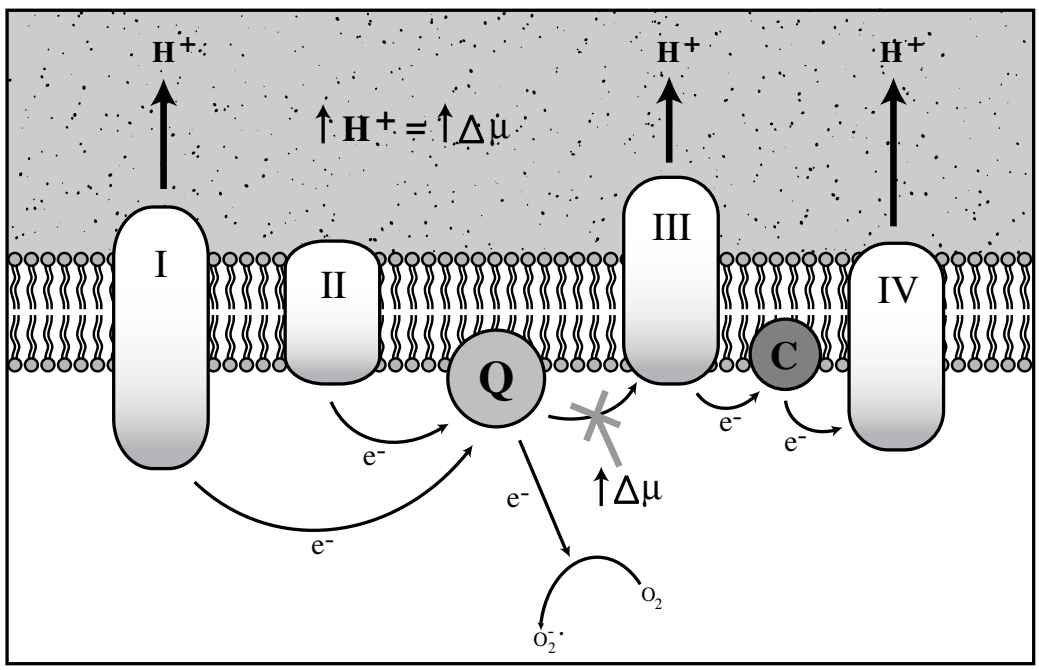

FIG. 2. The mitochondrial proton gradient (Δμ) is increased as complexes I, III, and IV of the electron-transport chain pump H^+ into the cytosol, the result of an overactive Krebs cycle. A significantly increased proton gradient inhibits electron transfer from CoQ (Q) to complex III, inducing ROS production in the mitochondria. $O_2^{-\cdot}$, superoxide.

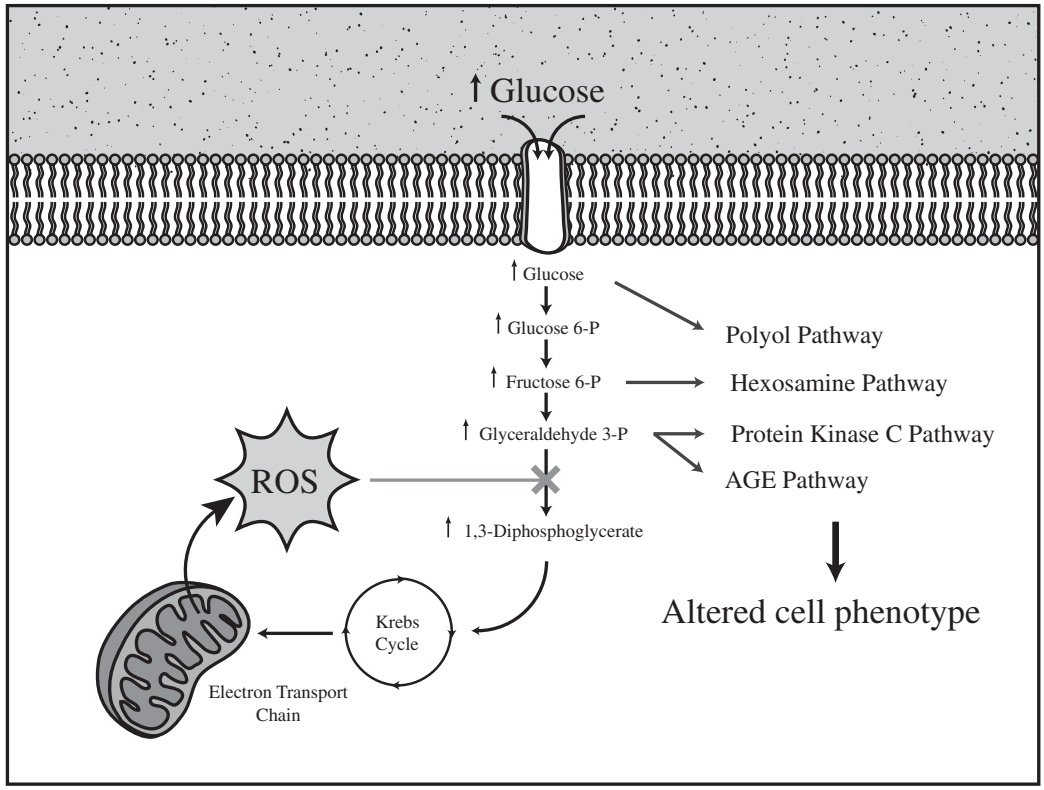

FIG. 3. Increased intracellular glucose entering the Krebs cycle increases ROS production. High levels of ROS inhibit glycolysis and activate the polyol, hexosamine, protein kinase C, and AGE pathways, which lead to cellular damage in diabetes.

include natural antioxidants (vitamins C and E, CoQ, and α-lipoic acid) and more advanced, mitochondrial targeted compounds such as tricarboxylic acid cycle uncouplers and recombinant superoxide dismutases (SOD). To date, few large-scale double-blind trials of natural antioxidant use have been conducted in diabetics (41, 55). However, small-scale trials and animal studies have demonstrated a reduction in oxidative stress with antioxidant administration in diabetic subjects (2, 3, 54), although neovascular function was not assessed. Because natural antioxidants are not specifically targeted to mitochondria and are structurally hydrophobic, very high doses were required to produce a significant reduction in circulating ROS. However, newer strategies exist to deliver antioxidants directly to the mitochondria by covalent attachment to a triphenylphosphonium cation through an alkyl chain (16, 34, 35), which may overcome these limitations. By linking to these lipophilic cations, antioxidants can pass lipid bylayers easily and accumulate inside the mitochondria. *In vitro* studies in human fibroblasts and *in vivo* mouse experiments have been promising, demonstrating 80-fold greater concentrations of covalently modified vitamin E moieties in the mitochondria compared with endogenous levels (29, 30, 50).

Because the majority of ROS is produced intracellularly by the electron-transport chain (10), an artificial uncoupler to reduce mitochondrial membrane potential and suppress ROS production is another strategy that has been examined. *In vitro* studies in endothelial cells overexpressing uncoupler protein-1, a specific uncoupler of oxidative phosphorylation, demonstrated a significant reduction in ROS production (28). Although aimed primarily at weight loss, trials in obese patients with the artificial uncoupler 2,4-dinitrophenol (DNP) also lowered oxidative stress levels (25). DNP acts as a protonophore, allowing H^+ to return to the mitochondrial matrix from the cytosol. This reduces membrane potential and eliminates hyperglycemia-induced inhibition of the electron-transport chain. However, no studies have been done in diabetes specifically, and the high toxicity (severe hyperthermia followed by death if overdosed) creates a narrow therapeutic window (25).

Perhaps the most intriguing therapy under development is exogenous delivery of SOD (MnSOD) or a suitable mimetic (43, 44). SOD enzymes are metalloproteins that catalyze dismutation reactions to detoxify superoxide anions. In these reactions, a series of electron transfers occur in which lone electrons are removed from superoxide molecules and transferred to reversible reducing agents (the metalloprotein construct). Interestingly, these compounds were first tested in humans in the 1970s, as potential therapies for rheumatoid arthritis and osteoarthritis (22). Orgotein, the commercially available form, was prescribed for Crohn's disease, Peyronie's disease, multiple arthritic conditions, and reduction of acute side effects after chemotherapy and radiation (18, 27, 32). However, immunologic complications prompted orgotein's removal from the market in the 1980s. Aside from immunologic reactivity, early SOD mimetics were also limited by large size (limiting cell permeability), short circulating half-life, and expense (44). However, multiple studies in the past

decade have proven the *in vitro* efficacy of similar agents in reducing oxidative stress in diabetes and vascular disease through administration of exogenous recombinant SOD and transgenic overexpression of SOD (24, 36, 42). Although currently available SOD preparations are not able to overcome the immunologic complications of their predecessors, recombinant alternatives are under development that promise to address these shortcomings (48).

Potential uses for these compounds would ideally include systemic administration to target oxidative dysfunction in both mature and progenitor cell types, but may ultimately be limited by toxicity. This may prompt the use of these agents to selectively treat autologous endothelial progenitor cells *ex vivo* followed by reinfusion to restore pathways of neovascularization associated with diabetic vascular complications. Alternatively, the topical delivery of antioxidant agents to diabetic wounds may prevent localized ROS overproduction, ameliorating the impairments in tissue repair caused by diabetes.

CONCLUSION

Although the relationship between oxidative stress and diabetes is only partially understood, research to date has proven an association strong enough to warrant the development and use of antioxidant therapy in diabetic patients. Because of the significant impact of vascular complications and their suboptimal treatment with glucose control alone, antioxidant therapy remains attractive. Therapies aimed at oxidative stress reduction in endothelial cells and EPCs, the component cells of angiogenesis and vasculogenesis, provide a new strategy to reverse neovascular impairments in diabetes. Furthermore, measurement of the antioxidant and ROS status in undiagnosed patients might provide a diagnostic marker for disease and treatment guidance. Of the antioxidant therapies presently under consideration, the high efficacy described with SOD mimetic therapy seems the most likely candidate to reach clinical application. Regardless of the specific agent, dismutase therapy in some form could easily be envisioned as a frontline therapy in diabetics, augmenting conventional glucose management. As ROS production has been shown to be a primary upstream regulator of a broad spectrum of diabetic impairment, intervention would be expected to have a broad spectrum of effects, potentially reversing damage in other organ systems as well.

ABBREVIATIONS

AGE, advanced glycosylation end products; CoQ, coenzyme Q; DNP, 2,4-dinitrophenol; EPC, endothelial progenitor cell; MnSOD, manganese superoxide dismutase; ROS, reactive oxygen species; SOD, superoxide dismutase; VEGF, vascular endothelial growth factor.

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