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Effects of Free Radicals in Diabetes

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Abbreviations:

AGEs: advanced glycosylated end-products
HDL: high density lipoprotein
$HO^{\bullet}$: hydroxyl radical
IDDM: insulin-dependant diabetes mellitus
H_2O_2: hydrogen peroxide
LDL: low density lipoprotein
NO: nitric oxide
NOS: NO synthase
NIDDM: noninsulin-dependent diabetes mellitus
PGG_2: Prostaglandin G_2
PGH_2: polyunsaturated fatty acid
TNF: tumor necrosis factor
VLDL: very low density lipoprotein

Outline

Abstract

Diabetes mellitus is a metabolic disease characterized by hyperglycemia. It usually associates with a series of late complications, such as vascular and metabolic abnormalities, during its development. Type I and type II diabetes are two major types of this disease. The oxidative stress is greatly increased due to prolonged exposure to hyperglycermia in diabetes. Increased free radical generation, which results from increased non-enzymatic glycosylation, glucose autoxidation, and alterations in polyol pathway activity, may exert a modulator effect on the level of oxidative stress in diabetes. Reduced capacities of antioxidant defense systems in diabetes also increase the oxidative stress. Endothelial dysfunction, increased oxidation in lipid and lipoprotein and damage in long-lived proteins are some sequences of increased intracellular oxidative stress. Understanding the role of free radicals in the pathogenesis of diabetes will be useful for the treatment and prevention of diabetes and its complications. This paper will review the current progress in understanding of the correlation between free radicals and diabetes.

Introduction

Almost 8 million people in the united states have been diagnosed with diabetes mellitus, and 7 million more have it but do not know it [1]. Diabetes mellitus is a very complex chronic disease with syndrome of hyperglycemia [2]. It usually leads to a series of late complications; for example, vascular complications caused by endothelial cell dysfunction in diabetes. This disease is generally subclassified into two major types: 1) Type I is insulin-dependent diabetes mellitus (IDDM) which is totally dependent on exogenous insulin. 2) Type II is non-insulin dependent diabetes mellitus (NIDDM) which can be treated with dietary changes, exercise and oral medication [2].

The cause of diabetes mellitus is not fully understood. Recently, increasing evidence suggests that free radicals formation are involved in the pathogenesis of diabetes and the development of diabetic complications [3, 4]. The oxidative stress is significantly increased in diabetes because prolonged exposure to hyperglycermia increases the generation of free radicals and reduces capacities of antioxidation defense systems [3]. This paper will review the mechanisms of free radicals in the development of diabetes, study the effects of free radicals on diabetes and its complications, and propose future direction and experiments.

Generation of free radicals in diabetes

Hyperglycermia, the primary clinical manifestation of diabetes, has been accepted as being essential for the development of diabetic complication. Many evidence have indicated that some biochemical pathways strictly associated with hyperglycermia (non-enzymatic glycosylation, glucose antoxidation, polyol pathways) can increase the production of free radicals (Figure 1) [5].

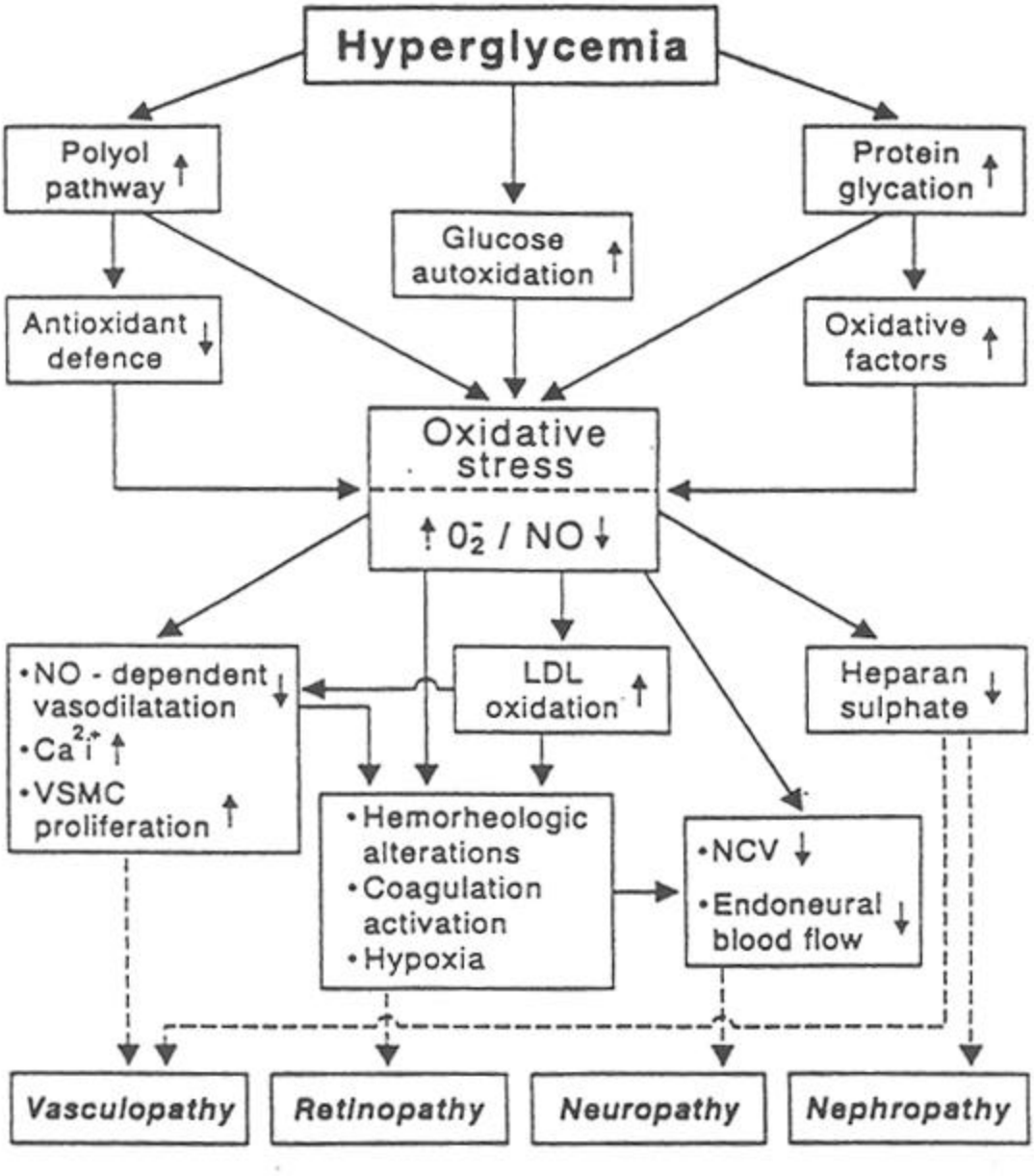

Figure 1. Possible links between hyperglycemia-induced oxidative stress and diabetic complications. Solid lines indicate experimentally established links. A genetic predisposition through reduced antioxidant status may be present (dashed lines). NCV, nerve conduction velocity; VSMC, vascular smooth muscle cell [5].

Free radicals generated by non-enzymatic glycosylation

Non-enzymatic glycosylation of protein ensues exposure to hyperglycemia. Initially, glucose undergoes a nucleophilic addition reaction with proteins to form the Schiff base. Formed early glycosylation product, ketomine is chemically reversible and thus is dissociated when blood glucose level return to normal. However, it subsequently undergoes an Amadori compound (Figure 2) [6]. Further reactions, rearrangements, dehydration and cleavage irreversibly results in the formation of brown, insoluble, crosslinking complexes called advanced glycosylation end-products (AGEs).

The Amadori products have been implicated in the formation of H_2O_2 in vitro [7]. Amadori products could form H_2O_2 via two pathways (Figure3) [7]. One pathway is the 1,2-enolization pathway, which lead to 3-deoxyglucosone formation under anaerobic conditions. In the presence of a suitable electron acceptor, however, enolization would occur to form H_2O_2 and glucosone [7]. The other pathway is 2,3-enolization pathway, which leads to 1-deoxyglucosone and the putative 1,4-deoxyglucosone [7]. Under oxidative conditions, however, the 2,3-enediol is thought to generate H_2O_2 and carboxymethyllysine [7]. 3-deoxyglucosones has been known to be a major and highly reactive intermediate in the non-enzymatic glycosylation and a potent cross-linker responsible for the polymerization of proteins to AGEs.

AGEs tend to accumulate on long-lived macromolecules in tissues. Cross-linking AGE-protein with other macromolecules in tissues results in abnormalities of cell and tissue functions [8]. In addition, AGEs contribute to increased vascular permeability in both micro- and macro- vascular structure by binding to a specific macrophage receptor [8]. This process induced the synthesis and secretion of cytokines such as TNF and IL-1, which causes endothelial dysfunction and induces free radicals.

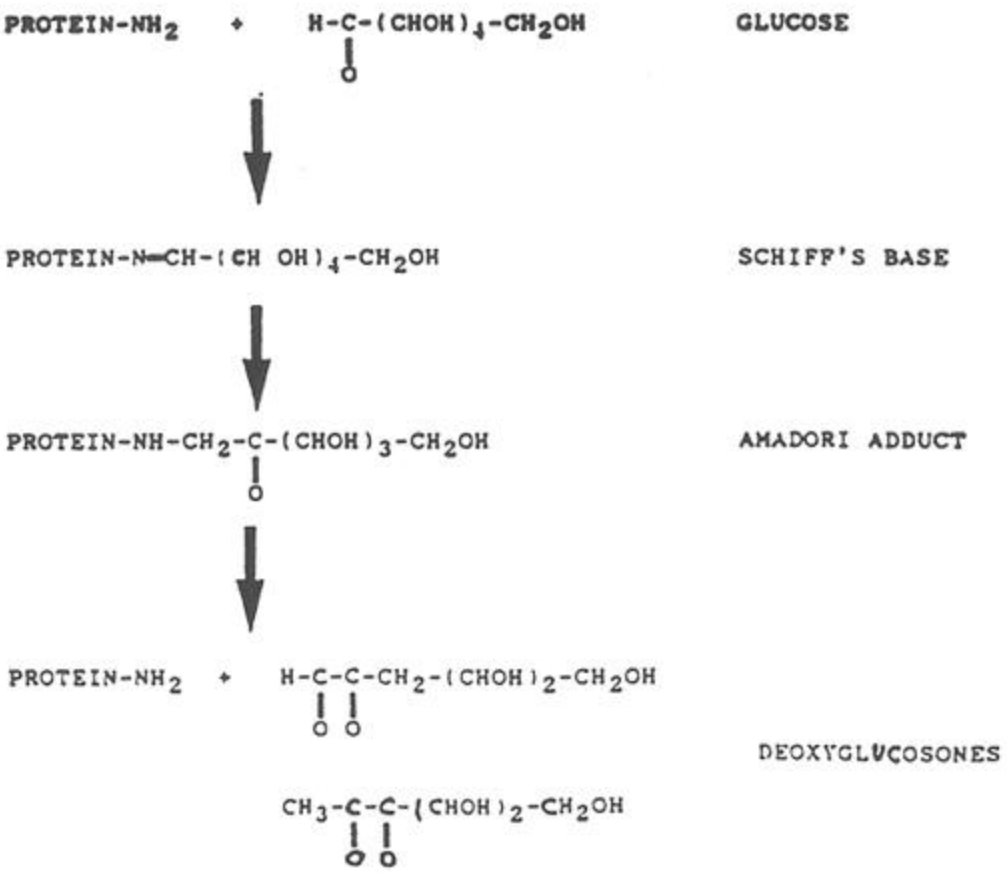

Figure 2. Non-enzymatic glycosylation. The addition of glucose to protein followed by rearrangements and dehydrations. The deoxyglucosones react with protein to form AGEs [6].

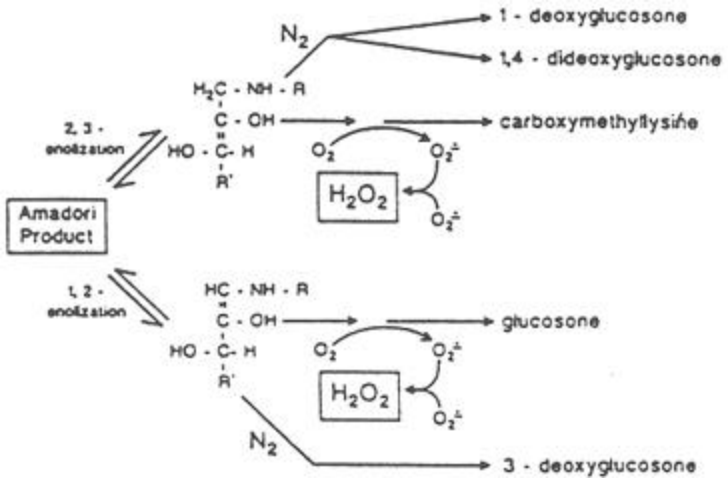

Figure 3. Degradation of Amadori product and H_2O_2 formation. H_2O_2 formation can be generated via both 1,2- and 2,3-enolization and oxidation of the enolate anion [7].

Free radicals generated by glucose autoxidation

In addition to direct glycosylation reactions, monosaccharides and fructose-lysine can spontaneously reduce molecular oxygen under physiological conditions [10]. The reduced oxygen products formed in the autoxidative reaction are superoxide, hydroxyl radical, and hydrogen peroxide. All can damage lipids, as well as proteins, through cross-linking and fragmentation. Free radicals also accelerate the formation of advanced glycosylation end-products, which in turn generate more free radicals. This process is called as glucose autoxidation [11]. Although the potential importance of this process in vivo is only indirect and has been inferred from in vitro experiments, there is some evidence in vivo that transition-metal chelating agents can prevent autoxidation in animal diabetes [12].

Free radicals generated by polyol pathways

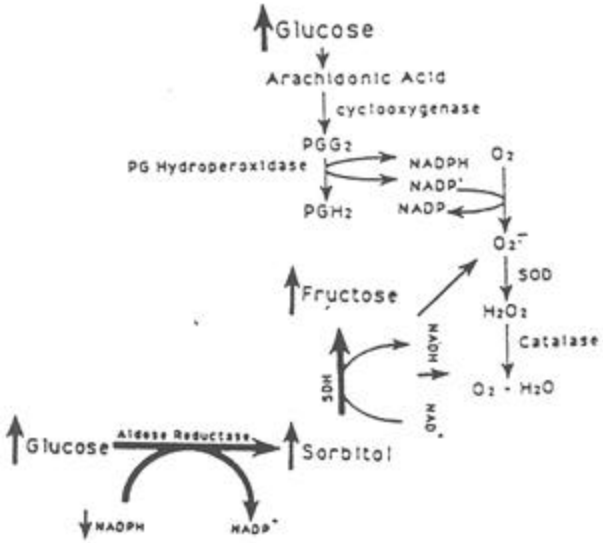

Figure 4. The polyol pathway: aldose reductase catalyzes the reduction of glucose by NADPH to sorbital which can, in turn, be oxidated to fructose by sorbitol dehydrogenase (SDH) leading to redox imbalance (NAD^+/NADH ratio). An increase in NAD^+/NADH ratio is linked to $O_2^{\cdot-}$ formation via the reduction of PGG_2 to prostaglandin H_2 (PGH_2) by prostaglandin hydroperoxidase that use NADH or NADPH as a reducing cosubstrate [14]

Free radicals can also be generated by polyol pathway [5]. Exposure to elevated glucose levels increase intracellular sorbitol and fructose content due to aldose reductase and sorbitol dehydrogenase activity (Figure 4) [11]. Oxidation of sorbitol to fructose is coupled to reduction of $NAPD^+$ to NADPH. An increase in NAD^+/NADH ratio is linked to $O_2^{\cdot-}$ formation via the reduction of prostaglandin G_2 (PGG_2) to prostaglandin H_2 (PGH_2) by prostaglandin hydroperoxidase that use NADH or NADPH as a reducing cosubstrate [11].

Reduced antioxidant capacity in diabetes

In addition to the increased generation of free radicals in diabetes, impaired generation of naturally occurring antioxidants also result in increased oxidative injury by failure of protective mechanisms [5]. Antioxidant defense system appears to be compromised in diabetic patients. It has been demonstrated that reduced scavenging of

free radicals by SOD [15] and lake of GSH [16] and ascorbic acid [17] are associated with diabetic vascular pathology. Reduced other antioxidants, such as vitamin E, uric acid, and reduced activity of catalase and GPx are also found in diabetes [18].

The mechanism by which the antioxidant reserve is reduced is not clear. Protein damage due to the protein glycosylation may be a mechanism that lowers the activities of primary antioxidant enzymes [15]. In addition, GSH deficiency may result from depletion of NADH in polyol pathway [4].

Free radicals in the pathogenesis of IDDM

Evidence for free radicals in the pathogenesis of IDDM

It is believed that IDDM diabetes is caused by the autoimmune beta cell destruction in pancreatic islets [19]. Beta cells normally secrete insulin in response to the increase of serum glucose. The destruction of beta cells results in deficiency and finally total loss of insulin secretion. What factors may trigger this autoimmune islet damage is an autroversy concerning. Increasing evidence suggest that free radicals play as one of factors in the beta cells damage. These evidence include 1) hydrogen peroxide, nitric oxide, and superoxide are toxic to the human, pig, and rat Islets in vitro [19]. 2) Alloxan and streptozotocin are two most commonly used drugs in developing the diabetes animal models due to their ability to selectively damage the insulin-secreting beta cells of pancreatic islets and induce impairment of islet glucose oxidation and of glucose-induced insulin secretion. It has been demonstrated that streptozotocin releases nitric oxide in isolated pancreatic islets [20] and alloxan stimulates superoxide generation in beta cells [2]. 3) Vitamin E suppressed the nitric oxide toxicity in the pancreatic islet cells in vitro

[21]. All these evidence points to the potential role of free radicals in the beta cell destruction.

The mechanisms of free radicals in beta cells destruction

Beta cells are prone to be destroyed by free radicals because of the low antioxidant enzyme nature [21]. Immune-effect cells such as macrophages, T cells, nature killer cells and B cells are believed to produce free radicals that causes damage to beta cells [22]. There are two mechanisms of free radicals in beta cells destruction.

First, infiltration macrophages produce superoxide as primary source of free radicals. The superoxide can be further converted to more active radical, hydroxyl radical which attacks cellular membrane and cause DNA breaks [3]. The consequence of DNA breaks leads cells death if cells fail to repair the damage. Also, the activation of DNA repair enzymes, especially the activation of poly (ADP-ribose) synthetase, deplete the NAD levels in cells, inhibiting proinsulin synthesis and, in addition, causing cells more sensitive to free radicals [3]. NAD and Na supplement can increase cellular NAD level so that elevates the efficiency of DNA repair and prevent decrease of proinsulin level [23].

Second, cytokines are released by T cells, macrophages, NK cells in the insulitis and induce the formation of intracellular free radical causing selective damage to beta cells [24]. Interleukin 1 (IL-1) is the major factor in the damage of beta cells. In addition, interferon γ (ITF γ) and tumor necrosis factor (TNF) are released macrophages during the insulitis [24]. These cytokines induce intracellular free radicals in endothelial cells, fibroblasts, and beta cells [24]. Two types of free radicals are induced from these cells [24]. One type is superoxide. The toxicity of superoxide via hydroxyl radicals

could be the same as above. Another type is NO. IL-1 βcan induce the production of nitric oxide synthase (NOS), which is the enzyme in charge of the synthesis of NO. Therefore, IL-1 induces NO formation. Many evidence have suggested that NO results in directly inhibitory effect on beta cells mitochondria function [24], thus NO can directly destroy beta cells.

Free radicals in the pathogenesis of NIDDM

Comparing with IDDM, the aetiology of NIDDM is far more diversity. It is only known that NIDDM is a heterogeneous disorder, that is, very different pathologic events result in the same clinical symptom [25]. Genetic abnormalities, or environmental factors, or obesity, which may induce beta cells malfunction and / or insulin resistance, can cause mild hyperglycemia which further develops to NIDDM. Some studies indicate that there are alternations in free radicals generation and antioxidant enzymes. In patients with essential hypertention and NIDDM, the oxidant stress is high and the plasma levels of No are low [26]. It has been shown that the mean lipid peroxide value and mean plasma glutathione peroxidase activity were significantly higher in women diabetic patients than in control [26]. The plasma level of NO and the activities of antioxidant enzymes were measured [26]. The plasma malondialdehyde (MDA) and NO levels, measured as its stable metabolite nitrite in the plasma were significantly low in patients with NIDDM (Table 1). The activities of SOD, glutathione peroxidase, glutathione reductase and vitamin E levels were significant low in the plasma in NIDDM, with no significant change in catalase activity (table 2). The free radical activity in NIDDM patients was increased as measured by the markers of free radicals activity [27].

Table 1. MDA and NO levels in patients with NIDDM (means ± SD) [26]

	Plasma MDA levels (nmole)		Plasma nitric oxide levels (μmol L^{-1} nitrite)	
	Fasting	Post-prandial	Fasting	Post-prandial
Control (n = 15)	1.57 ± 0.30	1.54 ± 0.44	0.90 ± 0.34	0.94 ± 0.28
NIDDM (n = 20)	2.15 ± 0.66*	2.42 ± 0.56*	0.56 ± 0.30*	0.57 ± 0.24*

*As compared with control, $p < 0.05$.

Table 2. Plasma levels of various anti-oxidants in patients with NIDDM (means ± SD) [27]

Anti-oxidant	Control (n = 15)		Patients with NIDDM (n = 20)	
	Fasting	Post-prandial	Fasting	Post-prandial
SOD (U mL^{-1})	8.50 ± 4.8	9.48 ± 2.17	2.61 ± 1.46*	4.45 ± 2.0*
Catalase (kU mL^{-1})	6.14 ± 0.02	6.15 ± 0.02	6.13 ± 0.03	6.13 ± 0.01
Glutathione peroxidase (NADPH oxi min^{-1} mL^{-1})	0.68 ± 0.07	0.70 ± 0.13	0.44 ± 0.12*	0.50 ± 0.07*
Glutathione reductase (U mL^{-1})	122.4 ± 32.1	153.6 ± 33.9	101.4 ± 22.5	92.7 ± 25.1*
Vitamin E (μg mL^{-1})	5.17 ± 1.61	5.53 ± 1.82	1.78 ± 1.57*	1.98 ± 1.28*

*As compared with control, $p < 0.05$.

Endothelial cells dysfunction by NO

Endothelial cells play an important role in vascular relaxation because they continuously produce NO by NO synthase (NOS) through incorporation of molecular oxygen into L-arginine, resulting in the formation of NO and L-citrulline [4, 28]. NO, as a potent endogenous nitrovasodilator, modulates vascular tone by increasing the production of cGMP in smooth muscle cells (Figure 5) [28]. Recent studies have shown that endothelium-dependent vasodilation is abnormal in diabetic patients (both IDDM and NIDDM) and this abnormality is caused by decreased release or activity of NO [29]. One of the most important determinants of NO bioavailability is the reaction of NO with reactive oxygen species. Many biochemical pathways associated with hyperglycemia, such as non-enzymatic glycosylation, glucose autoxidation and polyol pathway increase the production of free radicals. Superoxide anion has been implicated in the

physiological inactivation of NO. Superoxide anion exhibits endothelium-dependent vasoconstrictive properties by inactivation of basal release of NO and by stimulation of endothelium-derived vasoconstrictor prostanoids [30].

Prolonged hyperglycemia leads to an alternative metabolism of glucose through the polyol pathway [5]. The effect of an increased polyol pathway is an increased cytosolic NADH/NAD^+ ratio [30]. Such an altered redox state may influence the availability of tetrahydrobiopterin (BH_4), an essential cofactor for NOS [31]. During BH_4 depletion NOS is "uncoupled", leading to increased superoxide, rather NO production. In diabetic animal models BH_4 supplementation has been shown to improve impaired endothelium-dependent vasodialation [32].

The importance of increased polyol formation in diabetic endothelial dysfunction is supported by the observation that aldose reductase inhibitors restore endothelium-dependent relaxtions in diabetic animals. There are several mechanisms that explain this effect. Aldose reductase requires NADPH as a cofactor and, thus, an increased flux through the aldose reductase pathway leads to depletion of NADPH. Because NADPH is required for generation of NO from arginine, the depletion of NADPH is a possible explantion for impairment of endothelial cell function in diabetes (Figure 5). The endogenous antioxidant enzymes such as glutathione reductase also require NADPH, and NADPH depletion has been reported to increase generation of reactive oxidants during hyperglycemia [29]. Because glutathione is one of the most important protective factors against oxidative damage, its depletion is responsible for increased susceptibility to tissue injury.

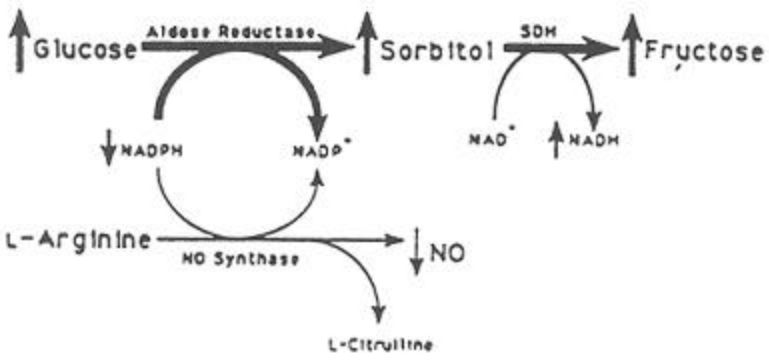

Figure 5. The polyol pathway: aldose reductase catalyzes the reduction of glucose by NADPH to sorbitol which can, in turn, be oxidized to fructose by sorbitol dehydrogenase (SDH) leading to redox imbalance ($NADH/NAD^+$ ratio). NO synthase requires NADPH as a cofactor; its depletion leads to reduced NO formation [30].

Defects produced by free radicals

Free radicals are able to produce a large amount of damages to biological components. For example, free radicals attack on DNA breaks. Free radicals can also react with proteins and lipids. For diabetic complications, the abnormalities of proteins and lipids are the major causes.

In diabetic patients, extracellular long-lived proteins, such as collagen, elastin, laminin, are the targets of free radicals. These proteins are modified to form glycoprotein via glycosylation pathways in diabetes due to hyperglycemia. The glycoproteins are futher broken into fragments by free radicals [33]. A higher amount of some cabornhydrate-derived oxidation products formed from oxidative breakage of glycoproteins are found in diabetic patients than in matched normal controls [34]. The modification of these protein and structural changes in tissues rich in these proteins such as lens, vascular wall, and basement membranes are associated with the development of complications in diabetes such as cataracts, microangiopathy, atherosclerosis and nephropathy.

Besides long-lived proteins, free radicals also cause oxidation of lipoproteins. Diabetes is almost always related to the changes in plasma lipoproteins. There are multiple abnormalities of lipoprotein metabolism in VLDL, LDLand HDL in diabetes. Principally, the modification in diabetic patients includes oxidaton of lipoproteins, mostly LDL. The oxidized lipoproteins are then rapidly internalized by macrophages, which in turn convert to cholesterol-loaded foam cells. The formation of the foam cells is a key mechanism in atherosclerotic lesion [17]. It was found that an increased level of oxidative lipoproteins in plasma from streptozotocin induced diabetic rats [35]. Treatment with antioxidants, vitamin E and probucool decrease the oxidation of lipoproteins without decrease of blood glucose [35].

In addition, lipid peroxidation induced by free radicals is another source for development of diabetic complication. Free radicals react with polyunsaturated fatty acid (PUFA) to form peroxides [39]. Peroxidation of PUFA results in the degradation of lipids and releases malnodialdehyde as final products. The breakdown of lipid components causes characteristic changes in lipid rich cell component. In a word, diabetes is accompanied by enhanced lipid peroxidation, and that hyperglycemia and accelerated oxidation is related.

Future directions and experiments

Antioxidant gene therapy

It was found that the activity of SOD and GPx was low in beta cells, rendering the beta cells more susceptible to free radical damage [2]. Normally, SOD performs the dismutation of superoxide to hydrogen peroxide. And GPx converts hydrogen peroxide, the product of superoxide dismutation, into water. So, in cells, SOD and GPx cooperate

together in the pathway where two ROS, superoxide and hydrogen peroxide, are turned into water. What is the exact role of SOD and GPx in preventing the beta cells from free radicals attack? Adenovirus-mediated and transient gene transfers are two ways to deliver foreign genes into quiescent somatic cells. Commonly, the viral DNA does not integrate into the host genome and is localized as nonreplicating extrachromosomal DNA within the nucleus [36]. Thus, adenovirus-mediated gene transfer can be considered a safe gene delivery system. Experiment 1 is designed to test the effects of double overexpression of MnSOD and GPx by adenovirus-mediated gene transfer on preventing the destruction of the pancreatic beta cells and ultimate development of diabetes.

Experiment 1: To investigate the effect of double overexpression of MnSOD and GPx on preventing the destruction of beta cells and ultimate development of diabetes.

Low-dose-streptozocin treatment has been used to induce diabetes by destruction of pancreatic beta cells [20]. A progressive decrease of SOD and GPx level in pancreatic beta cells was found with increasing periods of the streptozocin treatment [20]. So, it was suggested that the low activity of antioxidant protective enzymes is the reason for the high susceptibility of beta cells to streptozocin. The changes in pancreas antioxidant enzymes may reflect susceptibility of beta cells to free radical destruction. This study will focus on overexpression of MnSOD and GPx on preventing the destruction of the pancreatic beta cells and ultimate development of diabetes.

After using adenovirus-mediated gene transfer as a means of delivering the MnSOD and GPx cDNA to streptozotocin-induced diabetic rats, check the proteins level of MnSOD and GPx in beta cells by Western blot and the activity by Activity assay. The

overexpression of MnSOD and GPx beta cells will be exposed various free radicals for certain length of time, then the cell death will be assessed by Trypan blue dye exclusion and insulin secreting will also be checked by ELISA (enzyme-linked immunosorbent assay).

Free radical scavengers

The oxidative stress is greatly increased due to prolonged exposure to hyperglycermia in diabetes. Increased free radical generation, which results from increased non-enzymatic glycosylation, glucose autoxidation, and alterations in polyol pathway activity, may exert a modulator effect on the level of oxidative in diabetes. A delayed onset and a decreased incidence of diabetes mellitus were found when the rats were fed with a mixture of free radical scavengers [37]. What is the real effect of free radical scavengers in preventing the development of diabetes? Vitamin C and Vitamin E are the known free radical scavengers because they inhibit glucose autoxidation and reduces the covalent linking of glucose to serum proteins in vitro [38]. Most studies have found that people with diabetes mellitus have at least 30% lower circulating vitamin C and vitamin E concentrations than that of people without diabetes mellitus [45]. The cellular intake of vitamin C and vitamin E is inhibited by hyperglycemia and promoted by insulin [40, 41]. So, diabetic patients are particularly suitable for vitamin C and vitamin E supplementation. Experiment 2 is designed to test the effects of vitamin C and vitamin E supplementation on minimizing AGEs formation in vivo.

Experiment 2: To investigate the effects of vitamin C and vitamin E supplementation on minimizing AGEs formation in vivo.

Non-enzymatic glycosylation irreversibly results in the formation of advanced glycosylation end-products (AGEs) and glucose autoxidation accelerates this formation. Cross-linking AGE-protein leads to cells dysfunction. Also, AGEs increase vascular permeability that causes endothelial dysfunction. This study will focus on vitamin C and vitamin E supplementations minimize AGEs formation in vivo.

Using diabetic rats as animal model, supplement vitamin C and vitamin E in the food while control rats don't have these supplements. The formation of Hb-AGE will be a marker of hyperglycemic damage and measured by affinity chromatography.

Gene targeting approaches

In the diabetic status, hyperglycemia has been demonstrated to contribute to the elevated oxidative stress that in turn plays a role in the development of diabetic complications. One of the mechanisms is the altered activity of the polyol pathway, which consists of aldose reductase and sorbitol dehydrogenase. In this pathway, aldose redutase is the rate-limiting enzyme. Exposure of cultured endothelial cells to an elevated concentration of glucose diminished the glutathione-redox-cycle, and this could be prevented by inhibition of aldose reductase. So, inhibition or depletion of aldose reductase may have some effects on endothelial cell dysfunction. "Gene knockout" is the most frequent application of gene targeting approaches [42]. It is an event mediated by homologous recombination, resulting in the disruption of a specific gene [42]. Experiment 3 is designed to test the effects of the depletion of aldose reductase on the endothelial cell dysfunction in vivo.

Experiment 3: To investigate the effect of the depletion of aldose reductase on diabetic endothelial cell dysfunction in vivo.

Aldose reductase is a rate-limiting enzyme catalyzing the conversion of glucose to sorbital. During hyperglycemia, aldose reductase is activated and part of the glucose is metabolized to sorbital. As sorbital does not diffuse readily across cell membranes, it accumulates intracellularly and may cause damage [43]. Aldose reductase may be a target for prevention of endothelial cell dysfunction.

Two approaches will be used to achieve the aldose reductase depletion, one is to knockout yhe aldose reductase gene, and another is by using antisense RNA technique to inhibit aldose reductase gene expression. Then check whether or not aldose reductase depletion will restore endothelium-dependent relaxation in diabetic animals.

Summary

Diabetes mellitus is a chronic disease characterized by hyperglycemia with a lot of serious complications. Many evidence have shown that free radicals formation are involved in the pathogenesis of diabetes and development of diabetic complication. Oxidative stress is greatly increased in diabetes due to prolonged exposure to hyperglycemia. Non-enzymatic glycosylation, glucose autoxidation and polyol pathway increase the generation of free radicals. Reduced capacities of antioxidant enzymes also lead to increased oxidative stress in diabetes. Endothelial dysfunction, lipid and lipoprotein oxidation, and long-live proteins damage are the sequences of increased oxidative stress. Understanding the mechanisms of free radicals in diabetes will be useful for the treatment and prevention of diabetes and its complications.

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