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Understanding Diabetes From a New Perspective: The Role of Free Radicals

By

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Abbreviations:

AGE	Advanced glycation endproducts
AR	Aldose reductase
ARI	Aldose reductase inhibitor
DAG	Diacylglycerol
GR	Glutathione disulfide reductase
GSH	Glutathione
GSSG	Glutathione disulfide
IDDM	Insulin dependent diabetes mellitus
MnSOD	Manganese superoxide dismutase
NADPH	Nicotinamide adenine dinucloetide phosphate
NIDDM	Non-insulin dependent diabetes mellitus
$NO^{\bullet}$	Nitric oxide
NOS	Nitric oxide synthase
NOx	NADPH oxidase
PKC	Protein kinase C
SDH	Sorbitol dehydrogenase
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

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Abstract

Diabetes is a leading cause of death worldwide. It is caused by the deficiency of hormone insulin in the body causing improper utilization of glucose. Glucose accumulation in the blood causes hyperglycemia that in turn leads to several complications associated with diabetes. Several pathways (*e.g*. Hexosamine and polyol pathways, activation of protein kinase C *etc*) have been proposed to explain the mechanism by which hyperglycemia leads to vascular and other complications in patients. Apart from some classical explanations, many unconventional theories have been recently proposed suggesting a major involvement of oxidative stress in diabetes. This report summarizes the classical approach to explain diabetic complications and analyzes the role of oxidative stress and free radicals in the pathogenesis of the disease.

1. Introduction

Diabetes is a metabolic disorder responsible for numerous deaths each year around the world [1]. It has been reported recently that the risk for most cardiovascular diseases in the United States has decreased over the past 40 years, except, diabetes. Health care costs for diabetes are estimated to be nearly $100 billion per year in the United States [2].

Diabetes is associated with improper generation or utilization of the hormone insulin. Insulin is involved in the metabolism of glucose. The pancreatic cells (beta cells) produce insulin, which helps the cell to take up glucose produced by metabolism of food. Since, diabetes is associated with defects in insulin production, cells of diabetic patients cannot take up glucose effectively from the bloodstream. This leads to excessive glucose in blood, a condition called hyperglycemia [3] (**Figure1**). Hyperglycemia causes osmotic imbalances in the blood. To maintain this, the body demands more than normal water. Thus, excessive thirst is one of the early symptoms of the disease. Other common symptoms associated are excessive urination, extreme hunger, weight loss, irritable temper, fatigue, inefficient wound healing, dry skin and vision impairment [3].

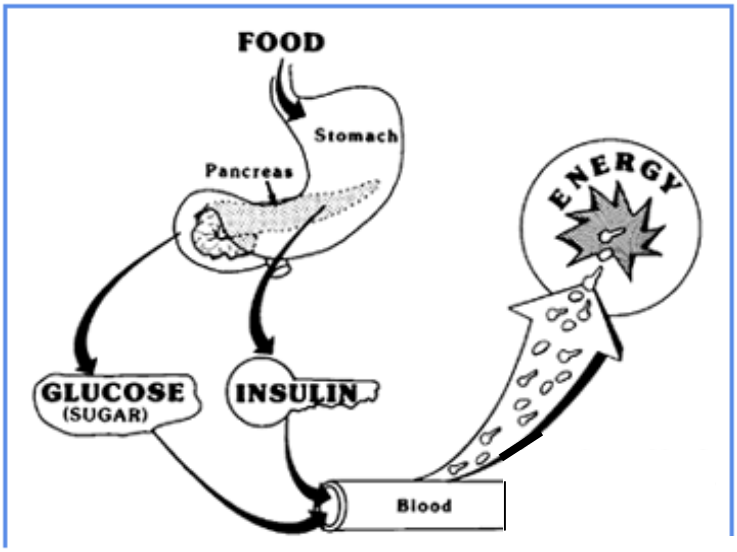

Figure 1: Insulin helps in assimilation of glucose produced by metabolism of food. Glucose can then be utilized by the cell to produce energy. During lack of insulin, Glucose cannot be taken up by the cell and accumulates in the blood [5].

There can be two types of diabetes, I and II. Type I diabetes is also called the "Juvenile diabetes". It accounts for about 10% of all diagnosed cases and is found in infants at birth. It is an autoimmune disease whereby the immune system of the body goes haywire and destroys its own pancreatic cells. This leads to insulin deficiency in the body. Thus, this type of diabetes is called insulin dependent diabetes mellitus (IDDM). The more commonly occurring diabetes is non-insulin dependent diabetes mellitus (NIDDM) or type II diabetes. This is usually found in adults and accounts for about 90-95% of diabetes cases [4]. In type II diabetes, pancreatic cells produce insulin, but either it's not produced in sufficient amounts, or it's not utilized properly. About 80% of the patients suffering from type II diabetes are overweight, have a sedentary lifestyle or a genetic history of the disease [5]. Type II diabetes is a progressive disease which can cause severe cardiovascular and other complications. Most of these complications can be avoided by dietary restrictions and proper exercise. It has been shown that physical activity of about 30 min/day significantly reduces the risk of cardiovascular diseases in diabetic patients [5]. Apart from the most common type I and type II diabetes, it has been observed that about 3-5% of pregnant women develop diabetes during pregnancy. This is also called "Gestational diabetes". It usually does not cause any birth defects, but increases the chances of these women developing diabetes at an older age [5].

2. Diabetic complications

In general, four pathways have been suggested to be involved in diabetic complications. These are [6]:

1. Increased advanced glycation end products (AGE)
2. Increased polyol pathway flux

3. Activation of protein kinase C
4. Increased hexosamine pathway flux

The common theme in most of these pathways is the induction of oxidative stress during hyperglycemia by a certain mechanism, which eventually leads to diabetic complications. Thus, irrespective of which pathway predominates, oxidative stress appears to be the underlying cause of majority of the diabetic complications.

2.1 Formation of AGE's

As discussed above, diabetes can cause hyperglycemia leading to a high sugar concentration in blood. Sone of these sugars such as glucose and fructose are reducing sugars. These reducing sugars can cause glycation of proteins leading to their inactivation (**Figure 2**). These glycated proteins formed as a result of this reaction are referred to as advanced glycation endproducts (AGE). Formation some of these AGEs can be used as marker for diabetes.

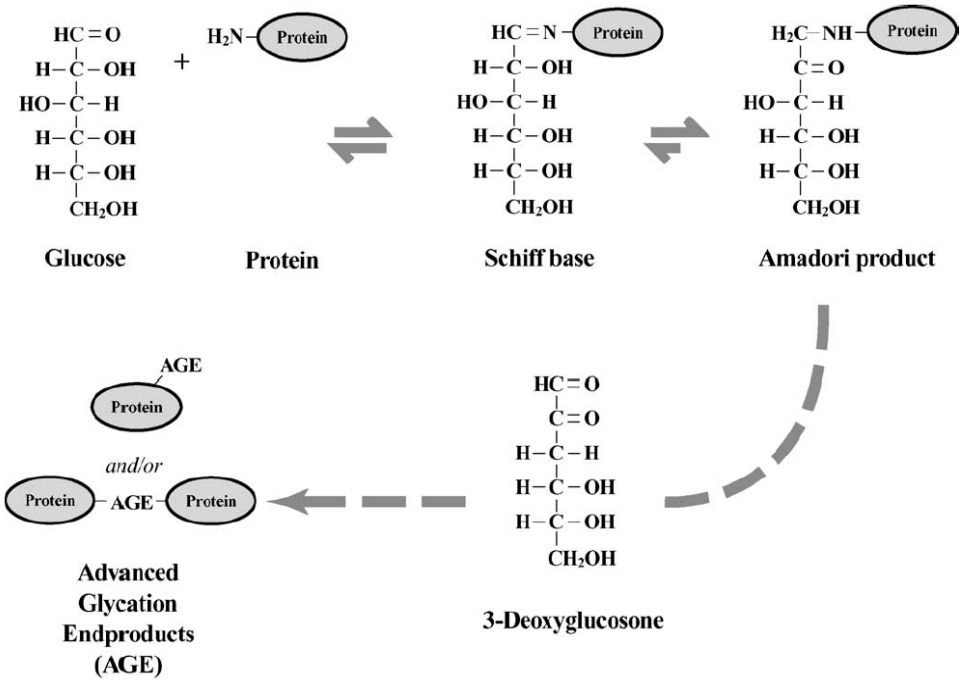

Figure 2: Formation of advanced glycation endproducts can lead to inactivation of certain important proteins. Adapted from [2].

AGEs can also be formed by autoxidation of glucose in the presence of transition metals and oxygen *via* an enediol intermediate (**Figure 3**). Thus, hyperglycemia leads to free radicals formation.

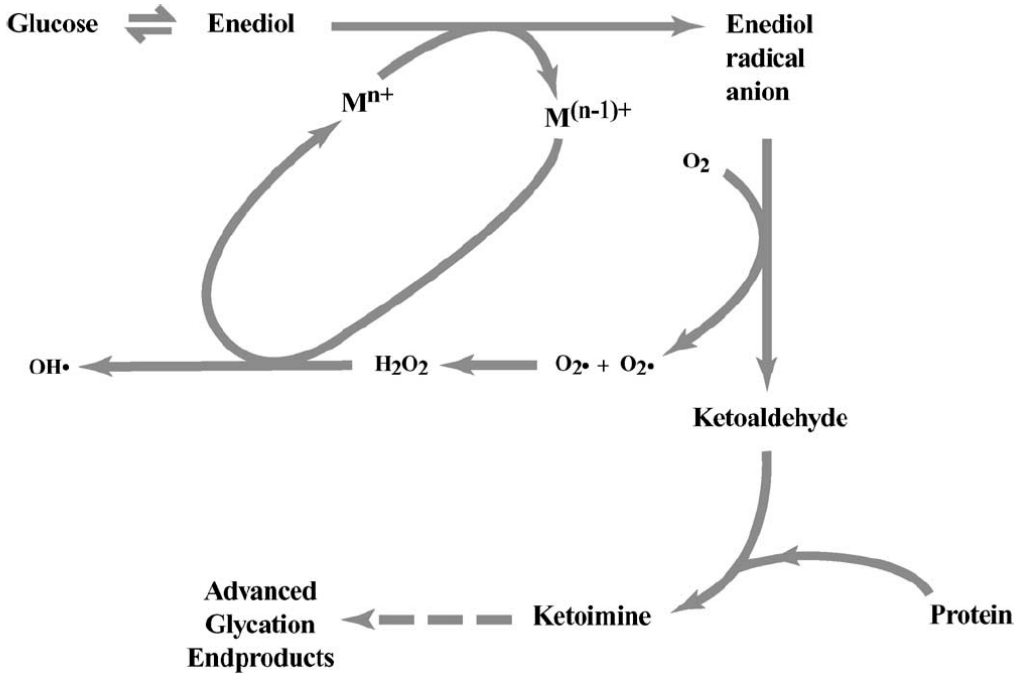

Figure 3: Alternative pathway for formation of AGE. Free radicals are formed in this process which can cause cellular toxicity. Adapted from [2].

Increased glycation and build up of AGE has been related to diabetic complications because they can alter enzyme activity, decreased ligand binding, modify protein half-life and alter immunogenecity. Glycation derived free radicals can cause fragmentation of protein, oxidation of nucleic acids and initiation of lipid peroxidation [7,8]

2.2 Polyol pathway

The polyol pathway is shown in **Figure 4** [9]. One of the first enzymes in the pathway is aldose reductase (AR) that converts glucose to sorbitol. It has been reported that AR inhibitors (ARI) prevent diabetic complications.

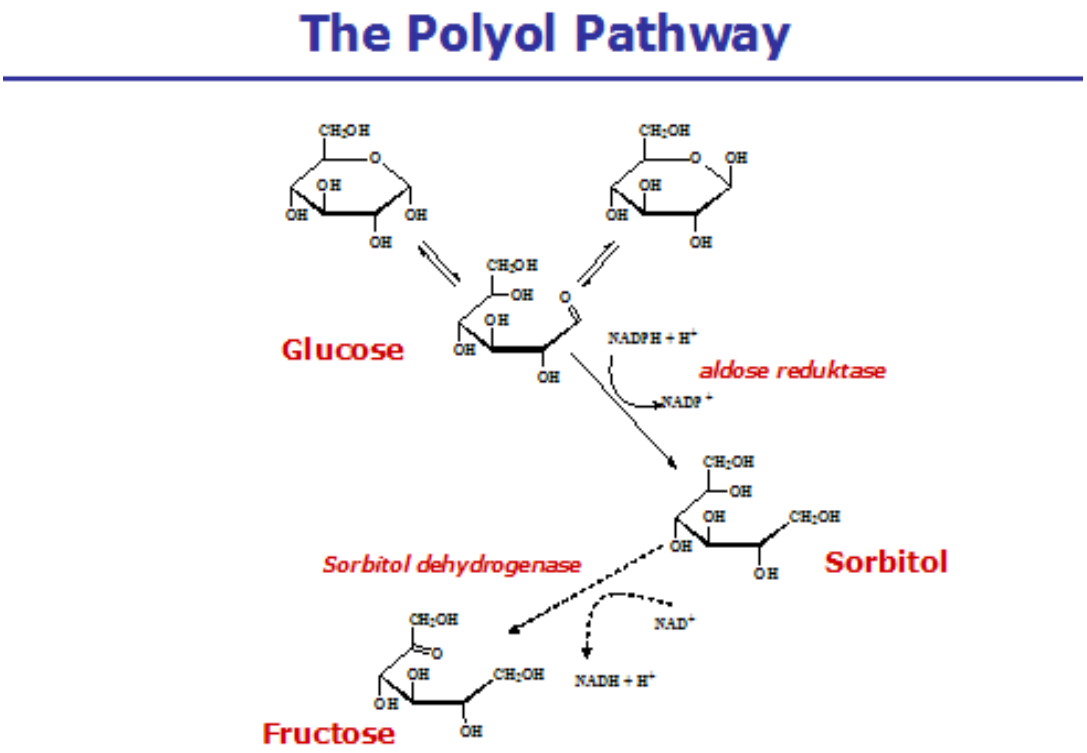

Figure 4: The polyol pathway. Aldose reductase and sorbitol dehydrogenase convert glucose to fructose. During hyperglycemina, flux through this pathway is increased as oppsed to glycolysis. Sugars such as fructose are more easily oxidized/glycated than glucose. This modified sugar can cause oxidative stress, leading to diabetic complications [7].

Furthermore, treatment with ARI leads to increase in GSH content of the cell, which in essential for cell survival. On meeting with oxidative stress, GSH is oxidized to GSSG. GSSG is eventually reduced back to GSH by the enzyme glutathione reductase (GR). The connection between the polyol pathway and GSH comes from the common cofactor, NADPH, for GR and AR [10]. It is proposed that during diabetes, the polyol pathway is upregulated, by the upregulation of AR. This leads to a competition for NADPH between AR and GR, leading to a loss of GSH. This makes the intracellular environment more oxidized, leading to oxidative stress, which in turn causes diabetic complications [11] (**Figure 5**).

2.3 Activation of protein kinase C (PKC)

Yet another mechanism by which hyperglycemia can lead to diabetic complications is by the activation of protein kinase C. Hyperglycemia causes *de novo* synthesis of diacylglycerol (DAG) which activates PKC. PKC controls activities of crucial enzymes such as cytosolic phospholipase A_2, Na^+/K^+ ATPase, and regulates several vascular functions such as permeability and contractility [12]. Most diabetic complications such as retinopathy and neovascularization are results of improper vascular functions. Thus, it is proposed that during hyperglycemia, activation of PKC through DAG causes these vascular alterations leading to diabetic complications (**Figure 6**) [13].

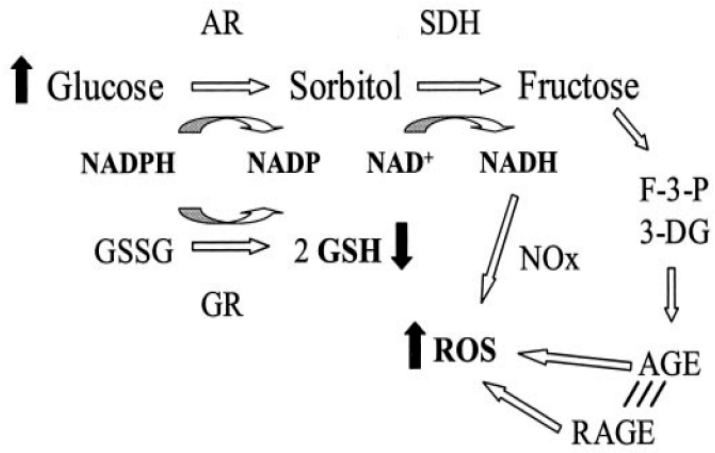

Figure 5 Role of GSH on induction of diabetic complications through ROS. Increase in polyol pathway causes decrease in GSH through competition reaction between AR and GR and an increase in NADH through the upregulation of SDH. Increased SDH causes increased NADH, which in turn induces NOx. NOx causes ROS production that is responsible for several diabetic complications [11].

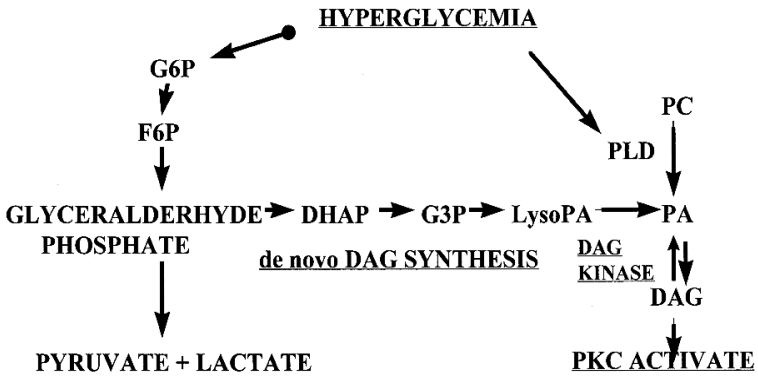

Figure 6: Activation of PKC through DAG. Activated PKC causes impaired blood flow and vascular abnormalities leading to diabetic complications such as retinopathy [13].

It has been reported that some of the diabetic complications related to vascularization, can be prevented or reduced using PKC inhibitors [14]. Also, antioxidants such as vitamin E have

shown to play a protective role, suggesting involvement of free radicals [15]. However, not much investigation has been carried out to explore the role of free radicals in PKC mediated diabetic complications.

2.4 Hexosamine pathway

Hexosamine pathway is shown in **Figure 7**. Hyperglycemia during diabetes increases the flux through hexosamine pathway. Overexpression of the first enzyme in the pathway, glutamine fructose 6-phosphate amidotransferase (GFA), has been shown to cause hypoinsulinemia, insulin resistance, and eventual diabetic complication [16].

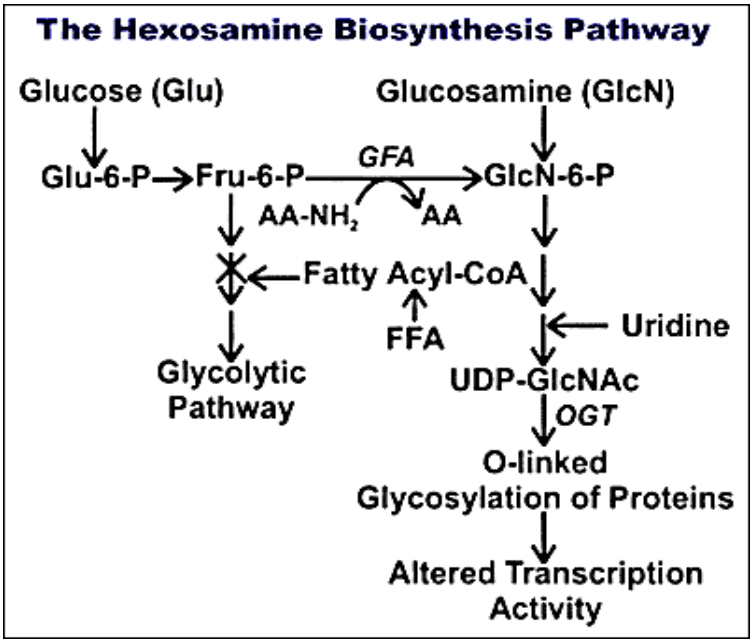

Figure 7: GFA is the most regulated and rate limiting enzyme in the pathway. Hyperglycemia leads to an increased flux through the hexosamine pathway as opposed to glycolytic pathway. This pathway eventually leads to formation of glycosylated protein products, which causes diabetic complications through AGE pathway. Adapted from [16]

2.5 The unifying hypothesis [12]

Since no pathway seems to explain pathogenesis of diabetes satisfactorily, a unifying hypothesis has been proposed. According to the unifying hypothesis, diabetic complications cannot be explained by any one pathway alone. Hyperglycemia, the root cause behind most of the diabetic complications, manifests its effect through the combination of the AGE, polyol, PKC/DAG and the hexosamine pathway (**Figure 8**). Each of these pathways do seem to suggest that oxidative stress has a major role to play.

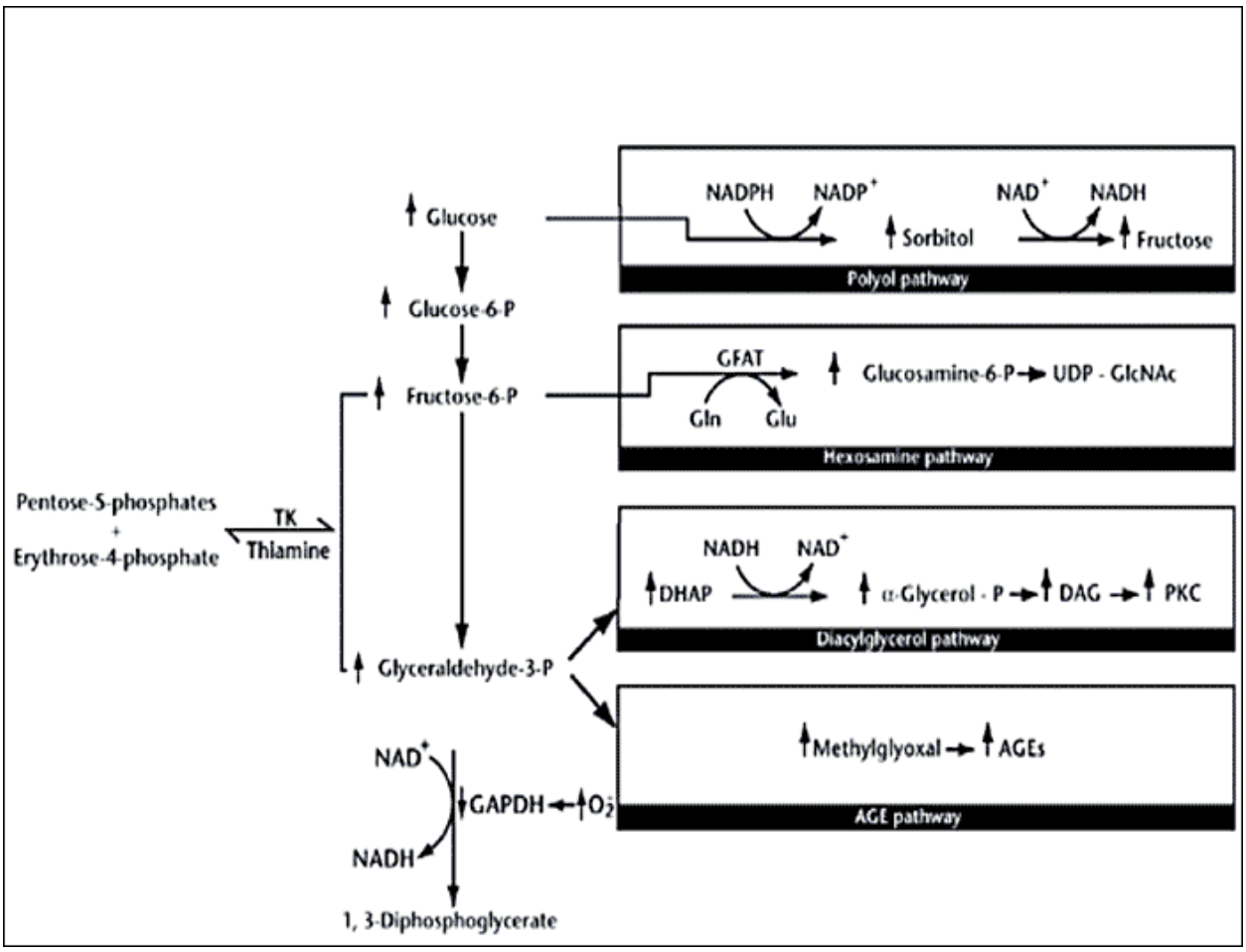

Figure 8: The unifying hypothesis for manifestations of diabetic complications. Each pathway is connected to glucose metabolism either directly or through one of the downstream metabolites [12].

2.6 Role of superoxide and nitric oxide in diabetic complications

Nitric oxide plays a key role in modulating endothelial function. Several isoforms of nitric oxide synthase (NOS) control the synthesis of nitric oxide. Inducible NOS (iNOS) is responsible for $NO^{\bullet}$ production in the endothelial cells [17]. It has been shown that hyperglycemia induces $NO^{\bullet}$ synthesis by iNOS and vasodilation would be expected [18]. However, certain reports suggest that hyperglycemia also causes overproduction of $O_2^{\bullet -}$ by the mitochondrial electron transport chain. Thus, $O_2^{\bullet -}$ quenches $NO^{\bullet}$ inhibiting its any possible

vasodilation effects. Reduced levels of $NO^{\bullet}$ have been reported in hyperglycemics [19]. Moreover, this leads to the production of the dangerous peroxynitrite species [20].

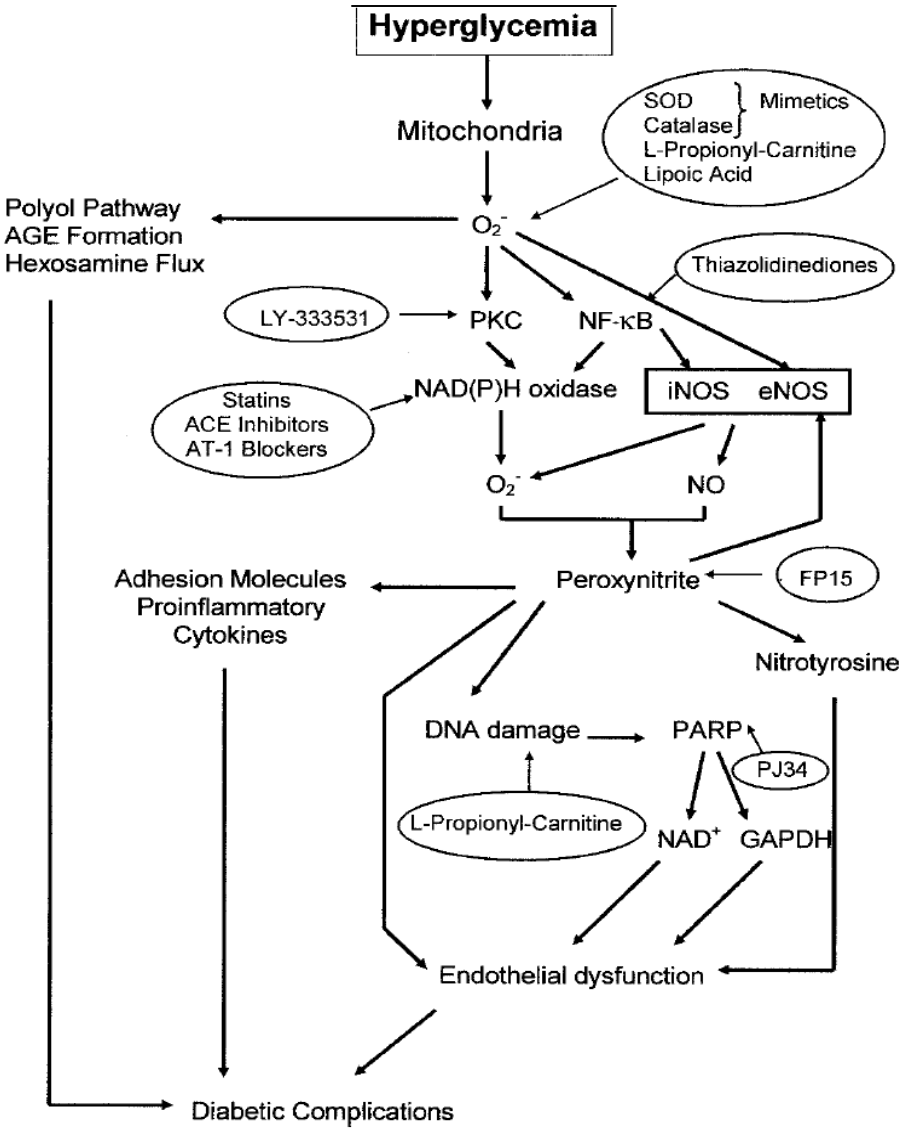

Figure 9: Role of superoxide and nitric oxide in diabetic complications. Superoxide can either directly cause diabetic complications by increasing the flux through the hexosamine, polyol or AGE pathways or indirectly through peroxynitrite formation *via* induction of iNOS and eNOS [21].

3. Are free radicals involved in diabetic pathogenesis?

Diabetes, its pathogenesis and epidemiology have been studied for several years. Unfortunately no cure for the disease has been found and it continues to be one of the leading causes of death in the world. One of the reasons for this is our yet incomplete understanding of the disease. Another reason is that diabetes manifests its symptoms through more than one pathway, thus making it difficult to treat the disease.

There is enormous evidence available to demonstrate the role of free radicals in several diseases [21]. More and more evidence is accumulating to show the involvement of free radicals in occurrence of diabetic complications. In fact, several scientists now believe that all the different pathways involved in diabetes eventually generate free radicals that then cause oxidative stress leading to DNA damage and subsequent diabetic complications [22] (**Figure 10**). There is

evidence to show that diabetic patients tend to have higher levels of lipid hydroperoxides and isoprostanes, which are markers of oxidation [23]. Diabetic patients also have a higher percentage of DNA damage and oxidized pyrimidines, which support the idea of high oxidative stress in diabetics. Altered purines also show a positive correlation with blood glucose level [24].

Moreover, several studies have shown that there is a decrease in the antioxidant enzyme levels (e.g. GPx, SOD, Catalase and GR) in diabetes patients [23]. An indirect evidence for the involvement of free radicals comes from the high levels of thiobarbituric acid reactive substance (TBARS) levels seen in diabetic mice. TBARS levels are lowered if these animals are treated with antioxidants such as vitamin C, E and β-carotene [25].

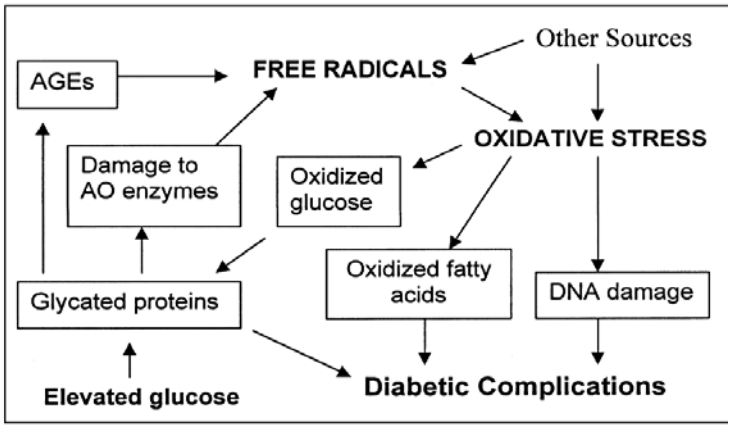

Figure 10: Most of the pathways studied to explain the occurrence of diabetic complications exhibit their effects through free radicals. Some products of hyperglycemia such as AGEs, can independently lead to diabetic complications as well. Thus, although free radicals are involved, they are only a part of the big picture. Adapted from [21]

Another straightforward evidence for involvement of free radicals is the beneficial effect of antioxidants such as vitamin E, selenium and α-lipoic acid in diabetes induced mice and even in a fraction of diabetes patients [26]. A point of controversy is the glutathione (GSH) level in mice with chemically induced diabetes. GSH level is found to be lower than normal in most organs, which supports the increased oxidative stress theory. However, in lens and kidney, GSH is actually higher than normal [28]. The GSH levels and their association with diabetic complications need further studies.

Thus, it is clear from the above discussion that oxidative stress is a key player responsible for several diabetic complications. Since the effects of oxidative stress are mostly mediated through free radicals, they are in understanding the various symptoms associated with diabetes.

4. Approaches to diabetic treatment

Currently, almost all treatments available for diabetes are symptomatic, meaning; they cure the symptoms of the disease but not the cause. Some of the possible treatments include physical exercise to keep weight in check, diet restrictions to keep carbohydrate intake in check and diabetes pills to bring down uncontrolled sugar levels if necessary. In some cases (especially Type I diabetes) insulin injections might be necessary [27]. However, it is important to realize that in diabetes, as in all other diseases, it is essential to treat the root cause of the disease rather than the symptoms. Therefore, several unconventional treatments such as antioxidant-based therapies are being proposed.

5. Understanding diabetes better: novel experiments

5.1 Experiment I

In **Figure 5**, we see the competition between AR and GR for NADPH cofactor. Since AR is overactive in most diabetes patients, this competition leads to a net loss of GSH. As discussed before, there is evidence to show that GSH levels are low in most tissues of the diabetic patients. Loss/deficiency of GSH eventually leads to oxidative stress.

Hypothesis

It would be worthwhile to see the effect overexpression of the enzyme γ-glutamylcysteine synthetase (γ-GCS). This enzyme is involved in GSH synthesis, and will lead to overproduction of GSH. This will help the animal in combating oxidative stress induced diabetic complications.

Proposed model

Diabetic mice may be created by alloxan or streptozotocin treatment. Assuming the polyol pathway and its mechanism to explain diabetic complications to be correct, these mice should have overproduction of the enzyme AR. A transient adenoviral transfection of γ-GCS to these mice should reduce some of the diabetic complications.

5.2 Experiment II

Figure 6 talks about the mechanism of PKC activation during diabetes. PKC activation is mediated through upregulation of DAG. Activated PKC then leads to endothelial dysfunction and diabetic complications. DAG kinase is the enzyme responsible for breakdown of DAG.

Hypothesis

Treatment of diabetes with DAG kinase mimetic should prevent PKC activation.

Proposed model

This again can be shown in a mouse model with chemically induced diabetes. Treatment with a DAG kinase mimetic should lead to downregulation of PKC. This can be easily seen on a western blot by probing for PKC expression. If the activation of PKC is mediated only through DAG, DAG kinase mimetics should lower PKC expression in a dose dependent manner. Thus, diabetic complications mediated by the PKC pathway should be less evident in these animals.

5.3 Experiment III

In **Figure 8**, we see the mechanism by which free radicals can possibly cause diabetic complications. The two key species appear to be superoxide and nitric oxide.

Hypothesis

If this mechanism is correctly understood, a concurrent overexpression of eNOS and MnSOD should at least partly prevent these complications.

Proposed model

This can be elegantly done in a mice model. The mice can be made diabetic using dibetogenic chemicals such as streptozotocin or alloxan. They can then be transfected to overexpress MnSOD (or orally given SOD mimetics) which will eliminate superoxide. Overexpression of eNOS will lead to and increased endothelial nitric oxide concentration. Since the superoxide is not available to quench it, together this will lead to an increase in $NO^{\bullet}$ bioavailability in endothelial cells causing vasodilation. This could possibly eliminate diabetic symptom, such as retinopathy.

5.4 Experiment IV

A direct evidence for the involvement of free radicals in diabetic pathogenesis can be provided by detection of the peroxynitrite species. It is proposed that simultaneous overproduction of superoxide and nitric oxide lead to the formation of peroxynitrite in diabetic patients.

Hypothesis

Peroxynitrite level in the cytoplasm of diabetes patient should be higher than peroxynitrite level in normal controls.

Proposed model

Peroxynitrite is an oxidizing species that can be measured by dichlorodihydrofluorescein (DCFH) fluorescence. We can take tissue samples (such as liver, kidney, blood) of diabetes patients. Separate cytoplasmic fraction from mitochondria, because peroxynitirte species is expected to form in the cytoplasm. Measure the oxidation of DCFH by measuring its fluorescence at 500 nm. If the model is correct, fluorescence intensity of diabetic tissues should

be higher than normal tissues. This will provide evidence in favor of the oxidative stress and free radical theory of diabetes pathogenesis.

6. Summary

Diabetes has been a major health problem worldwide, responsible for several deaths. The fact that it can be found in people of any age makes it a bigger threat. It is a complex disease because it can have multiple pathways leading to similar effects. Although a lot has been studied in this field, a lot more needs to be done. Four primary pathways have been proposed to explain diabetic complications. Although none of these fully explain all the complications suffered by

diabetic patients, they do improve our understanding of the pathogenesis of the disease. Furthermore, several new and unconventional pathways have been presented in the literature to try and unify the effects of all the classical mechanisms. One such proposal is the emphasis on the role of free radicals in diabetes. From a review of past literature, it is clear that free radicals are important mediators for developing diabetic complications. This is an area that has not been fully explored yet. However, there is evidence to suggest that free radicals play a crucial role in all the four primary pathways of causing diabetic complications. This opens research possibilities to explore antioxidant therapies.

Since science is never without any exceptions, treatment of diabetes patients with antioxidants has given mixed results. Whereas most patients show a decline of TBARS when treated with antioxidants, some patients do not have any effect. Thus, there have been controversies on this issue and it is reasonable to conclude at this point that antioxidants might be a good adjuvant to the already existing treatment for diabetes and diabetic complications, but may not be sufficient treatment modality by themselves.

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