INVOLVEMENT OF FREE RADICALS IN CARDIOVASCULAR COMPLICATION IN TYPE 2 DIABETES - A REVIEW

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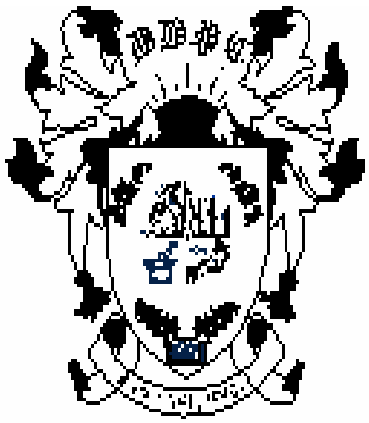

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In the past few decades, ***type 2 diabetes mellitus*** (T2DM) has rapidly increased in the world. It has been estimated that the number of diabetic patients will more than double within 15 years (1). Moreover, although T2DM was previously considered a slow-onset disease of middle-aged and older subjects, an emerging issue is the recent increase in diagnoses of T2DM and prediabetic conditions in children (2).

T2DM is mainly characterized by the development of increased morbidity and mortality for cardiovascular disease (CVD) (3), so that it has been suggested that diabetes may be considered a cardiovascular disease (4). However, CVD risk is elevated long before the development of diabetes (5). The close relationship between T2DM and CVD has led to the "common soil" hypothesis (6), postulating that T2DM and CVD share common genetic and environmental antecedents. One of the most important of these possible antecedents is considered insulin resistance. In genetically predisposed subjects, the combination of excess caloric intake and relatively scarce physical activity, with the likely consequence of obesity, can induce a state of resistance to the action of insulin (7). Insulin resistance is an important component of the metabolic syndrome, first described as a clinical syndrome in which the clustering of factors such as obesity, dyslipidemia, and hypertension leads to a substantial increase in CVD risk (8).

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. All forms of life maintain a reducing environment within their cells. This reducing environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.

Oxidative stress is considered to be the main cause for several chronic diseases including diabetes. Through hyperglycemia, hyperlipidemia, hypertension and possible iron dyshomeostasis, diabetes induces oxidative stress that causes damage to multiple organs, leading to various complications. Therefore, antioxidant therapy may be an interesting approach to prevent diabetes and diabetic complications.

Insulin resistance is also a crucially important metabolic abnormality in T2DM, and overt diabetes is thought to be preceded by a long period of insulin resistance, during which blood glucose is maintained near normal levels by compensatory hyperinsulinemia. When cells are no longer able to compensate for insulin resistance by adequately increasing insulin production, impaired glucose tolerance (IGT) appears. This condition is characterized by an excessive blood glucose concentration in the postprandial phase, with fasting glucose being in the normal range. Persistence of imbalance between caloric intake and expenditure eventually leads to overt diabetes, characterized by high glycemia in any condition whether fasting or postprandial. Studies currently performed in children point out the co-existence of obesity, insulin resistance, and -cell dysfunction, as occurs in classic older T2DM-prone subjects (9). The mix of overfeeding and sedentary habits has extended to children.

These 3 conditions, i.e., insulin resistance, IGT, and overt diabetes, appear to be associated, although to a variable degree, with an increased risk of CVD (10, 11). Recent trials have confirmed the hypothesis that lifestyle modifications, in terms of reduced caloric intake and

increased physical activity, can reduce the incidence of new cases of diabetes (12, 13). In other intervention trials, the same goal has been attained by means of several drugs.

There have been studies specifically aimed to demonstrate the ability of known antidiabetic drugs, i.e., metformin (12), troglitazone (14), and acarbose (15), to hamper the evolution from IGT to diabetes. Each of the 3 drugs was successful in that regard. Interestingly, although metformin and troglitazone were expected to act by abating insulin resistance, and secondarily hyperglycemia, acarbose lowers postprandial hyperglycemia by impairing carbohydrate absorption from the intestinal lumen without any direct effect on insulin resistance. It appears, then, that prevention of the development of diabetes is obtainable by simply lowering postprandial glycemic peaks. However, the picture is more intricate. In studies aimed to reduce the incidence of cardiovascular disease in high-risk populations by means of calcium channel blockers (CCBs), angiotensin-converting enzyme (ACE) inhibitors, angiotensin-1 (AT-1) receptor antagonists, and statins, all of which are devoid of any effect on glycemia, a significant reduction of new cases of diabetes has been incidentally discovered (16-20). It appears that in the prevention of diabetes, a direct action on insulin resistance is not a requisite, nor is the reduction of postprandial hyperglycemia. The questions now is what type of effect do CCBs (21), ACE inhibitors (22), AT-1 receptor antagonists (23), and statins (24) have that are responsible for the prevention of diabetes? Do these compounds share any mechanism of action with the aforementioned antidiabetic drugs? It has been shown that CCBs, statins, ACE inhibitors, and AT-1 receptor antagonists have a strong intracellular "preventive" antioxidant activity, and it has been suggested that many of their beneficial ancillary effects, such as a decrease in cardiovascular mortality not fully accounted for by hypotensive or lipid-lowering effects, may be caused by this property (21-24). If we consider that glitazones are intracellular antioxidants, too(25), and that postprandial hyperglycemia itself produces an oxidative stress (26), so that acarbose (an inhibitor of intestinal glucose absorption) and glinides (ie, repaglinide, nateglinide, and metiglinide, which restore the first phase of insulin secretion) may be expected to reduce oxidative stress by specifically lowering postprandial hyperglycemia, then the antioxidant effect is the only known property that all of these drugs have in common.

Because evidence suggests that overnutrition, insulin resistance, IGT, diabetes, and CVD share in common the presence of an oxidative stress (27-29), in this article oxidative stress generation is proposed as the common persistent pathogenic factor mediating the appearance of insulin resistance as well as the passage from insulin resistance to overt diabetes, via IGT, while producing the increased cardiovascular risk condition typical of prediabetic and diabetic subjects by favoring atherosclerotic complications. This hypothesis may help us understand why diverse therapeutic interventions, which have in common the ability to reduce oxidative stress, can impede or delay the onset of diabetes and CVD.

OVERFEEDING TO INSULIN RESISTANCE: THE ROLE OF OXIDATIVE STRESS

The most important tissues involved in the pathogenesis of insulin resistance are muscle and adipose tissue. When caloric intake exceeds the energy expenditure, the substrate-induced increase in citric acid cycle activity generates an excess of mitochondrial NADH (mNADH) and reactive oxygen species (ROS) (30). To protect themselves against harmful effects of ROS, cells may reduce the formation of ROS and/or enhance ROS removal. Prevention of ROS formation is accomplished by preventing the build-up of mNADH by inhibiting insulinstimulated nutrient uptake and preventing the entrance of energetic substrates (pyruvate, fatty acids) into the mitochondria. Controversy exists as to whether free fatty acid (FFA) or glucose is the primary

fuel source in the overnourished muscle and adipose tissue. In either case, an influx of substrates into the citric acid cycle generates mitochondrial acetyl-CoA and NADH (30). Acetyl-CoA derived either from glucose through pyruvate or from beta-oxidation of FFA, combines with oxaloacetate to form citrate, which enters the citric acid cycle and is converted to isocitrate. NAD┌ -dependent isocitrate dehydrogenase generates NADH. When excessive NADH cannot be Dissipated by oxidative phosphorylation (or other mechanisms), the mitochondrial proton gradient increases and single electrons are transferred to oxygen, leading to the formation of free radicals, particularly superoxide anion (31) (Figure 1). The generation of excessive NADH may be prevented in several ways, one of which is the inhibition of FFA oxidation (32). An increase in intracellular FFA, in turn, leads to reduced GLUT4 translocation to the plasma membrane, resulting in resistance to insulinstimulated glucose uptake in muscle and adipose tissue (33-35). In this setting, insulin resistance may be considered a compensatory mechanism that protects the cells against further insulinstimulated glucose and fatty acid uptake and therefore oxidative damage.

Many studies support this hypothesis: in vitro studies and in animal models, antioxidants have been shown to improve insulin sensitivity (36). Several clinical trials have demonstrated that treatment with vitamin E, vitamin C, or glutathione improves insulin sensitivity in insulin-resistant individuals (36, 37), although there is evidence from molecular biology studies to support the possibility that oxidative stress alters the intracellular signaling pathway inducing insulin resistance (28). The recent findings that insulin resistance is associated in humans with reduced intracellular antioxidant defense also support this hypothesis (38).

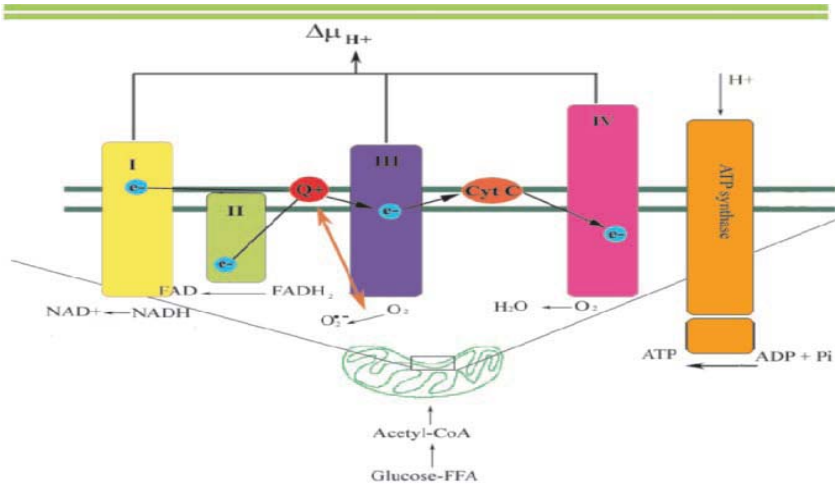

Figure 1. Possible mechanism of increased superoxide generation on mitochondrial electron transport chain during FFA and glucose overload. FFA and glucose overload increases the generation of AcetylCoA, which, in turn, increases the production of electron donors from the tricarboxylic acid cycle (NADH). This increases the membrane potential ($\Delta\mu H^+$), because protons are pumped across the mitochondrial inner membrane in proportion to electron flux through the electron transport chain. Inhibition of electron transport at complex III by increased $\Delta\mu H^+$ increases the half-life of free radical intermediates of coenzyme Q, which reduce O2 to superoxide. Pi indicates inorganic phosphorus.

OXIDATIVE STRESS PATHOGENIC FACTOR FOR THE DYSFUNCTION OF BETA AND ENDOTHELIAL CELLS

It is a reasonable hypothesis that what happens in muscle and fat cells may also occur in other cells, particularly in cells and endothelial cells. Moreover, these cell types may be particularly affected by overfeeding. These cells are notably not dependent on insulin for glucose uptake, which here is via facilitative diffusion instead of insulin-regulated glucose transporters. Therefore, if overfed, they cannot downregulation the influx of nutrients by means of insulin resistance, and must allow intracellular concentrations to increase further.

Many studies have suggested that cell dysfunction results from prolonged exposure to high glucose, elevated FFA levels, or a combination of both (28). Cells are particularly sensitive to ROS because they are low in free-radical quenching (antioxidant) enzymes such as catalase, glutathione per-oxidase, and superoxide dismutase (39). Therefore, the ability of oxidative stress to damage mitochondria and markedly blunt insulin secretion is not surprising; (40) for example, it has been demonstrated that oxidative stress generated by short exposure of -cell preparations to H_2O_2 increases production of p21 and decreases insulin mRNA, cytosolic ATP, and calcium flux in cytosol and mitochondria (31).The key role of increased glucose metabolism in producing impaired cellfunction through oxidative stress has recently been confirmed. Intracellular ROS increased 15 minutes after exposure to high glucose, and this effect was blunted by inhibitors of the mitochondrial function (41). Glucose-induced insulin secretion was also suppressed by H_2O_2, a chemical substitute for ROS (41). Interestingly, the first phase of glucose-induced insulin secretion could be suppressed by 50 M H_2O_2. H_2O_2 or high glucose suppressed the activity of glyceraldehydes 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme, and inhibitors of the mitochondrial function abolished the latter effects. These data suggest that high glucose concentrations induce mitochondrial ROS, which suppresses the first phase of glucose-induced insulin secretion, at least in part, through the suppression of GAPDH activity (41).

These results have been confirmed in vivo. In subjects with normal glucose tolerance, glutathione infusion failed to affect beta-cell response to glucose (42). In contrast, glutathione significantly potentiated glucose-induced insulin secretion in patients with IGT (42). Furthermore, in the latter group studied in the condition of hyperglycemic clamp, glutathione infusion significantly potentiated the cell response to glucose when plasma glucose levels varied between 10 and 15 mmol/L (42). Impaired insulin secretion has been associated with an FFA-induced increase in ROS, both in vitro (43, 44) and in vivo (45) Interestingly, it has been reported that both FFA and glucose may impair insulin secretion in cells by activating uncoupling of protein 2 (44, 46). In the case of hyperglycemia, it has been shown that such activation is accomplished by hyperglycemia-induced superoxide formation in mitochondria (46). Therefore, as glucose and FFA overload is present during increased caloric disposal, it is possible that the combination with high glucose will maximize cell toxicity. This hypothesis is supported by recent studies showing that when either isolated islets or HIT cells were exposed to chronically elevated glucose and FFA levels, there was a distinct decrease in insulin mRNA and the activation of an insulin-gene reporter construct (47). In other studies, co-culture of islets with high levels of glucose and palmitate resulted in almost complete impairment of glucose-stimulated insulin secretion, despite partially sustained stored insulin (44). Recent studies have suggested that cell lipotoxicity is enhanced by concurrent hyperglycemia and that oxidative stress may be the mediator.

The response-to-injury hypothesis of atherosclerosis states that the initial damage affects the arterial endothelium in terms of endothelial dysfunction (50). Notably, today's evidence confirms that endothelial dysfunction, associated with oxidative stress, predicts cardiovascular disease (51, 52). Insulin resistance is associated with impaired endothelial function (53). Glucose and FFA overload may be supposed to influence endothelial cells, as well as cells, producing an endothelial dysfunction through an oxidative stress. Indeed, many studies show that high glucose concentrations induce endothelial dysfunction. In vitro, the direct role of hyperglycemia has been suggested by evidence that arteries isolated from normal animals and subsequently exposed to exogenous hyperglycemia exhibit attenuated endothelium-dependent relaxation (54). Consistently, in vivo studies have also shown that hyperglycemia directly induces, in diabetic subjects and nondiabetic subjects, endothelial dysfunction (55, 56). The role of free radical generation in producing the hyperglycemia-dependent endothelial dysfunction is suggested by studies showing that in vitro57 and in vivo, (58, 59) the acute effects of hyperglycemia are counterbalanced by antioxidants.

Recent studies demonstrate that a single hyperglycemia induced process of overproduction of superoxide by the mitochondrial electron transport chain seems to be the first and key event in the activation of all other pathways involved in the pathogenesis of endothelial dysfunction in the case of hyperglycemia (60, 61). Superoxide overproduction is accompanied by increased nitric oxide generation, caused by eNOS and iNOS uncoupled state (62), a phenomenon favoring the formation of the strong oxidant peroxynitrite, which in turn damages DNA (61). DNA damage is an obligatory stimulus for the activation of the nuclear enzyme poly (ADP-ribose) polymerase (61). Poly (ADP-ribose) polymerase activation in turn depletes the intracellular concentration of its substrate NAD, slowing the rate of glycolysis, electron transport, and ATP formation, and produces an ADP-ribosylation of the GAPDH (61). These processes result in acute endothelial dysfunction. Convincingly, FFA may work the same way:(28) FFA increases oxidative stress generation in humans (46) and induces endothelial dysfunction, which can be reversed by antioxidants

FROM INSULIN RESISTANCE TO IGT: THE ROLE OF OXIDATIVE STRESS

Initially, insulin resistance is compensated by hyperinsulinemia through which a normal glucose tolerance is preserved. Deterioration to IGT occurs when insulin resistance increases further and/or the compensatory insulin secretory response decreases. An increase in insulin, FFA, and/or glucose levels can increase ROS production and oxidative stress, as well as activate stress-sensitive pathways (28) this, in turn, can worsen both insulin action and secretion, thereby accelerating the progression to overt type 2 diabetes. IGT, i.e., postprandial hyperglycemia with fasting glycemia in the normal range, is a risk factor for increased cardiovascular mortality (64) many studies show that postprandial hyperglycemia is associated with oxidative stress generation (64). A loss of early-phase insulin response is a common event in subjects with impaired glucose metabolism (65). This alteration may not simply be a marker of the risk of diabetes, but rather an important pathogenic mechanism causing excessive postprandial hyperglycemia (66). In response to intravenous glucose, insulin secretion is biphasic. The first phase is a rapid release of insulin into the bloodstream in response to the ingestion of carbohydrates or a mixed meal (7). The rapid increase in portal blood insulin concentration and the avid binding of the hormone to its receptors on liver cell membranes account for a prompt suppression of endogenous glucose production and a reduced rate of increase in plasma glucose concentrations (65).

In experiments performed in animals and humans, the selective abolition of early insulin secretion in healthy subjects resulted in IGT, excessive glycemic excursions, and possible hampering of the thermic effects of ingested carbohydrates (7, 65). In nondiabetic subjects, the loss of early insulin secretion is a determinant for the subsequent development of diabetes (65).

The critical role of the early-phase insulin response in determining postprandial hyperglycemia is supported by the demonstration that glucose tolerance is improved by restoring the acute increase in plasma insulin concentrations after the ingestion of both glucose and a mixed meal (65). This amelioration of the glycemic profile can prevent late hyperglycemia and hyperinsulinemia. Oxidative stress contributes, in vivo, to specifically alter the early phase of insulin secretion because the latter can be restored by antioxidants (36). Moreover, it has been proposed that mitochondrial overproduction of free radicals is a potential mechanism causing impaired first phase of glucose-induced insulin secretion (41). Evidence indicates that postprandial hyperglycemia is directly implicated in the development of cardiovascular disease, whereas evidence linking fasting glycemia to diabetic complications is inconclusive as yet (64). Moreover, in many studies postprandial glycemia is a better predictor of the cardiovascular risk than HbA1c, which reflects both fasting and postprandial blood glucose levels (64). Postprandial glucose may be directly involved in cardiovascular complications through a toxic effect on the vascular endothelium, mediated by oxidative stress (26). This atherogenic effect appears to be independent of other cardiovascular risk factors such as hyperlipidemia.

FROM IGT TO DIABETES AND ENDOTHELIAL DYSFUNCTION

Repeated exposure to hyperglycemia and increased levels of FFA can lead to cell dysfunction that may become irreversible over time (67). In its initial stages, this damage is characterized by a reversible defective insulin gene expression (38). Glucose and lipid toxicity induce the gradual, time-dependent establishment of irreversible damage to cellular components of insulin production, and, therefore, to insulin content and secretion (65). Oxidative stress is convincingly the mediator of such damage (28). Recent studies in type 2 diabetic animal models report that the progressive reduction of islet cells is associated with excessive oxidative stress (68). In these animal models, when hyperglycemia is allowed to continue, a so-called glucotoxicity to cells impairs insulin secretion and eventually causes fatal islet cell injury, accelerating cell loss (68). Consistently, Japanese type 2 diabetic patients show a reduction of cell mass and evidence of increased oxidative stress-related tissue damage that is correlated with the extent of the cell lesions (69). Vascular function in diabetes mellitus has been studied extensively in both animal models and humans. Impaired endothelium-dependent vasodilation has been a consistent finding in animal models of diabetes induced by alloxan or Streptozotocin (70, 71). Similarly, studies in humans with insulin dependent and non-insulin-dependent diabetes have found endothelial dysfunction when compared with vascular function in nondiabetic subjects (72, 73). Strong evidence suggests that oxidative stress is the mediator of impaired endothelial function in diabetes (74).

THE POSSIBLE LINK BETWEEN OXIDATIVE STRESS AND INFLAMMATION IN INSULIN RESISTANCE, DIABETES, AND CVD

Although the concept of atherosclerosis as an inflammatory disease is now well established, line of evidence suggests that chronic inflammation may be involved in the pathogenesis of insulin resistance and T2DM (75). This lead to the hypothesis that inflammatory changes may be considered a common pathogenic step in all of these conditions (75). The concept that oxidative stress is the common factor underlying insulin resistance, T2DM, and CVD, and may explain the presence of inflammation in all these conditions. It is well recognized that inflammation is one manifestation of oxidative stress (76), and the pathways that generate the mediators of inflammation, such as adhesion molecules and interleukins, are all induced by oxidative stress (76). Interestingly, it has recently been proposed that the subclinical

Pro-inflammatory state observable in many conditions including atherosclerosis, cancer, and aging is caused by a mitochondrial overgeneration of free radicals (76). Moreover, the hypothesis is supported by in vivo studies, showing that FFA and glucose induce inflammation through oxidative stress, have a cumulative and independent effect, and that antioxidants reverse the phenomenon (78-81).

OXIDATIVE STRESS AS THE CONNECTION BETWEEN NUTRITION OVERLOAD AND DIABETES AND RELATED CARDIOVASCULAR COMPLICATIONS: THERAPEUTIC IMPLICATIONS

Available evidence leads to the hypothesis, summarized in Figure 2, that oxidative stress can be considered the clue to the association of overnutrition with the development of overt diabetes. It may also link the progressive cell failure to an increased cardiovascular risk, a prominent association in the clinical setting.

However, this hypothesis can also contribute to understand why different therapeutic strategies, apparently having in common only the ability to reduce oxidative stress, appear to simultaneously lead to decreased cardiovascular mortality and lower incidence of diabetes. If oxidative stress is the pathogenic mechanism leading from insulin resistance to overt diabetes, the ability of a drug to prevent or reverse oxidant stress can account for its clinical usefulness (21-25). Furthermore, the beneficial effect of controlling postprandial hyperglycemia on both the development of diabetes (15) and the prevention of cardiovascular disease (82) also supports this hypothesis, because it has been shown that in the postprandial state there is an oxidative stress generation, which is strictly dependent on the level of glycemia reached (83).

However, even convincing evidence is now available supporting the hypothesis that oxidative stress may play a key role in the development of both diabetes and CVD clinical trials with antioxidants, in particular with vitamin E, have failed to demonstrate any beneficial effect(84). On this matter, it has recently been suggested that antioxidant therapy with vitamin E or other antioxidants is limited to scavenging already formed oxidants and may, therefore, be considered a more “symptomatic” rather than a causal treatment for oxidative stress (85). According to the evidence discussed in this article, it is suggested that interrupting the overproduction of superoxide by the mitochondrial electron transport chain would normalize the pathways involved in the development of the oxidative stress (86). It might, however, be difficult to accomplish this using conventional antioxidants, because these scavenge ROS in a stoichiometric manner.

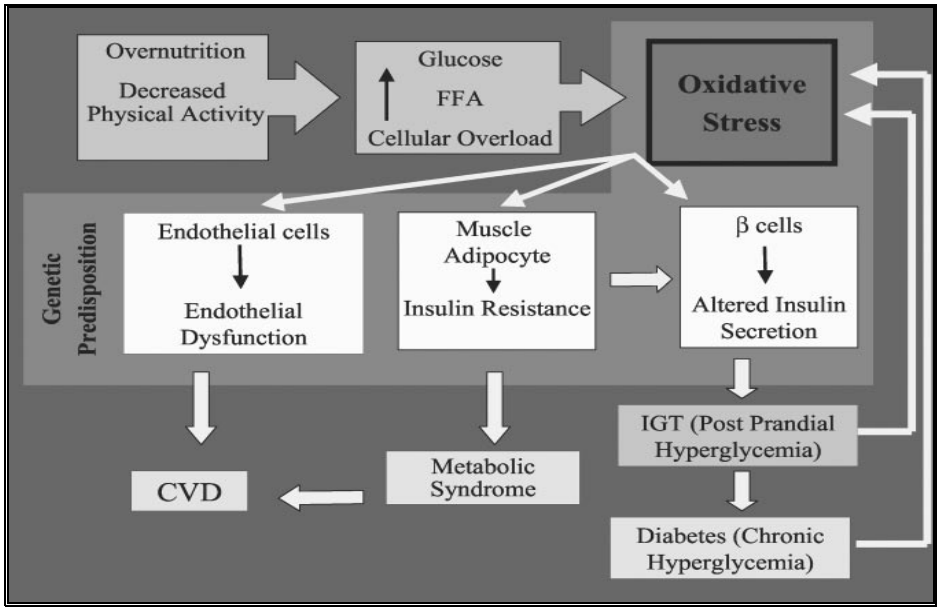

Figure 2. Overnutrition and decreased physical activity lead to increased glucose and FFA loads in cells. Their transformation in energy is accompanied by increased free radicals generation (oxidative stress). The muscle cells and adipocytes can protect themselves from this condition, producing a resistance to the action of insulin, aiming to reduce glucose and FFA penetration in the cells. Cells and endothelium are insulin-independent tissues. Glucose and FFA overload in these cells and cause oxidative stress, which in turn induces a dysfunction of both cells and endothelium. Endothelial dysfunction may lead to the development of cardiovascular disease; cell dysfunction is characterized by an alteration of insulin secretion. This last condition is worseed by the concomitant insulin resistance, which is a condition that requires increased insulin secretion to maintain plasma glycemia in a normal range. Cell dysfunction is particularly characterized by a decreased first-phase insulin secretion, which in turn produces the clinical picture of IGT. This last situation is clinically characterized by increased postprandial hyperglycemia. Postprandial hyperglycemia induces oxidative stress. The persistence of such condition produces an exhaustion of cells, leading to the overt diabetes. Oxidative stress produced both during IGT and overt diabetes may contribute to the development of cardiovascular disease. Moreover, the cluster of the risk factors that accompanies the insulin resistance also contributes to produce cardiovascular disease.

However, while waiting for more focused tools (87) CCBs (21), statins (22), ACE inhibitors (23), and AT-1 receptor antagonists (24) and glitazones (25), which have a strong ability to prevent intracellular oxidant activity, 86 seem to be valid options already available. This topic has been extensively reviewed (21, 85, 86). This concept is also summarized in Figure 3. In conclusion, a puzzle of many pieces of evidence suggests that free radical overgeneration may be considered the key in the generation of insulin resistance, diabetes, and cardiovascular disease. Even if a change in lifestyle remains the best preventive and therapeutic approach, many

new specific and causal antioxidants are being developed (85, 86) and may become important tools to oppose the increasing epidemic of diabetes, a real emergency in our future. Moreover, this concept can explain why treating cardiovascular risk with drugs such as CCBs, ACE inhibitors, AT-1 receptor antagonists, and statins may also prevent diabetes. Last but not least, because it has been demonstrated that insulin resistance is associated in humans with reduced intracellular antioxidant defense (38) and that diabetic subjects prone to complications may have a defective intracellular antioxidant response (88, 89), even what we call genetic predisposition to diabetes, as well as liability to its late complications, might be based on a deficient ROS-scavenging ability in cells and/or in target tissues such as endothelium.

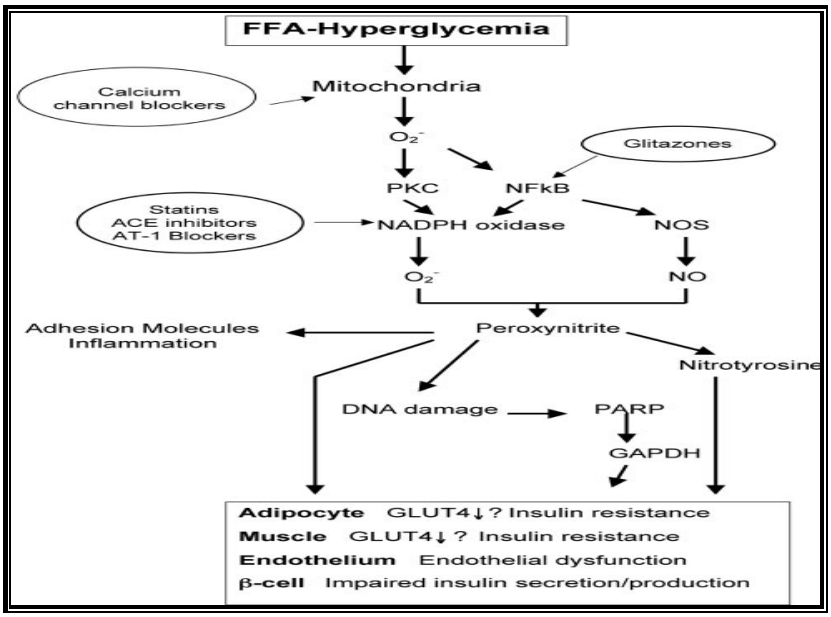

Figure 3. In the cells, hyperglycemia and FFA induce overproduction of superoxide at the mitochondrial level and nitric oxide overproduction through NOS, whereas PKC and NF-kB are activated and favor an overexpression of the enzyme NADPH. NADPH generates a great amount of superoxide. Superoxide overproduction, accompanied by increased nitric oxide generation, favors the formation of the strong oxidant peroxynitrite, which in turn damages DNA. DNA damage is an obligatory stimulus for the activation of the nuclear enzyme poly(ADP-ribose) polymerase. Poly(ADP-ribose) polymerase activation in turn reduces the GAPDH activity. This process results in the adipocyte and muscle in reduced GLUT4 expression and the subsequent insulin resistance, in endothelial cell in endothelial dysfunction, in cells in decreased insulin secretion/production. CCBs, statins, ACE inhibitors, ATI inhibitors, and glitazones may intervene at different levels in preventing this phenomenon.

Several recent studies have demonstrated that altered oxygen utilization and/or increased formation of reactive oxygen species (ROS) contribute to cardiovascular disease (CVD) progression. Many recent studies have suggested that oxygen derived free radicals may be important participants in a wide array of cardiac condition, and several clinical trials evaluating the use of antioxidants as therapeutics either have already been or are underway.

The initial suggestions of oxidative mechanisms during CVD were described in the acute setting of Myocardial infarction. These conditions are associated with a sudden reduction of coronary perfusion and oxygen availability, leading to altered myocardial metabolism, ROS production and cell death. Interestingly, ROS production and associated cellular damage is higher in cardiac tissues reperfusion relative to ischemic conditions. The acute paradigm of cardiac ischemia and reperfusion has provided insight into the mechanisms of ROS induced alteration of cardiac function and disease progression. In fact, it is now recognized that ROS may contribute to the progression of other more chronic cardiovascular condition that are not related to acute oxygen deprivation. In addition to cellular and/or tissue evidence of oxidative damage, elevated levels of oxidative stress marker are detected in several pathologic conditions of cardiovascular disorders, including hypertension, ventricular hypertrophy, atherosclerosis and cardiac heart failure.

In summary, despite the diverse etiology of cardiovascular conditions, the enhanced production of ROS and altered oxygen utilization is apparently a common phenomenon and a participant in disease progression. Further understanding of the events that contribute to these changes, and the cellular adaptations involved, may provide new opportunities for rational therapeutic strategies.

ACTIVATION OF OXYGEN:

One of the paradoxes of life on this planet is that the molecule that sustains aerobic life, oxygen, is not only fundamentally essential for energy metabolism and respiration, but it has been implicated in many diseases and degenerative conditions (90). A common element in such diverse human disorders as ageing, arthritis, cancer, Lou Gehrig's disease and many others is the involvement of partially reduced forms of oxygen. Our realisation of the significance of oxygen in disorders and stress-induced dysfunctions in cultivated plants is recent due in no small part to the difficulty in detecting and tracing oxygen molecules, to the multitude of forms and intermediates that oxygen can assume, and to the extreme reactivity and rate of the chemical reactions involved. As a consequence we often in our experiments can only look for the "footprints" of oxygen reactions in our attempts to determine cause-effect relationships in stress responses. The following chapter describes our current understanding of the general principles of activated oxygen.

Atmospheric oxygen in its ground state is distinctive among the gaseous elements because it is a biradical, or in other words it has two unpaired electrons. This feature makes oxygen paramagnetic; it also makes oxygen very unlikely to participate in reactions with organic molecules unless it is "activated". The requirement for activation occurs because the two unpaired electrons in oxygen have parallel spins. According to Pauli's exclusion principle, this precludes reactions with a divalent reductant, unless this reductant also has two unpaired electrons with parallel spin opposite to that of the oxygen, which is a very rare occurrence. Hence, oxygen is usually non-reactive to organic molecules that have paired electrons with opposite spins. This spin restriction means that the most common mechanisms of oxygen reduction in biochemical reactions are those involving transfer of only a single electron (monovalent reduction). Activation of oxygen may occur by two different mechanisms:

Absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one

of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins (Fig. 4). This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction). Since paired electrons are common in organic molecules, singlet oxygen is much more reactive towards organic molecules than its triplet counterpart.

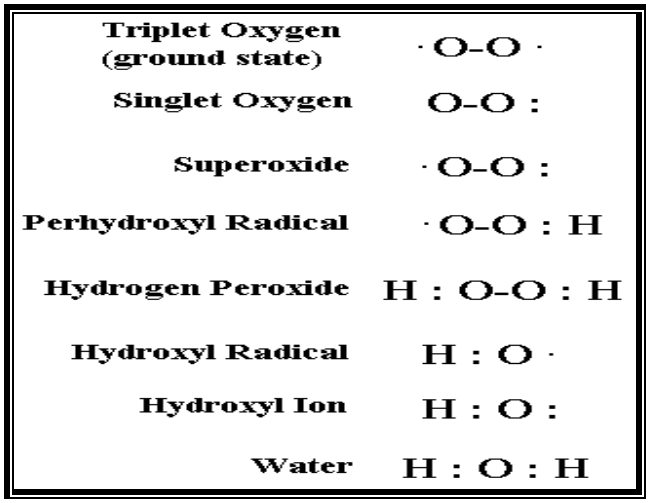

Figure 4: Nomenclature of the various forms of oxygen

The second mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide (O^{-}_{2}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and finally water according to the scheme shown in figure 2. The first step in the reduction of oxygen forming superoxide is endothermic but subsequent reductions are exothermic.

Superoxide can act as either an oxidant or a reductant; it can oxidise sulphur, ascorbic acid or NADPH; it can reduce cytochrome C and metal ions. A dismutation reaction leading to the formation of hydrogen peroxide and oxygen can occur spontaneously or is catalysed by the enzyme superoxide dismutase. In its protonated form (pKa = 4.8) superoxide forms the perhydroxyl radical (OOH) which is a powerful oxidant (91), but its biological relevance is probably minor because of its low concentration at physiological pH.

The univalent reduction of superoxide produces hydrogen peroxide that is not a free radical because all of its electrons are paired (Fig. 5). Very often the reduction products of oxygen are referred to by biologists as oxygen free radicals which is a misnomer because in chemistry a free radical is defined as an atom or molecule with an unpaired electron. It is more appropriate to refer to the intermediate reduction products of oxygen as activated not as free radicals because triplet oxygen (ground state) is a radical and hydrogen peroxide are not.

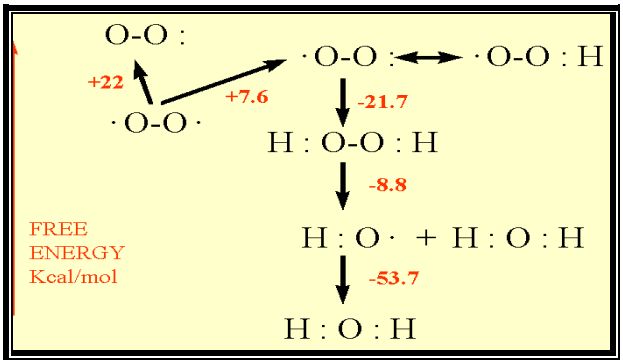

Figure 5: The activation states of oxygen. Non-activated oxygen is a biradical. From this triplet state it can be activated by either reversing the spin on one of the unpaired electrons to form the singlet state or by reduction. The first reduction reaction is endothermic forming superoxide. Subsequent reductions form hydrogen peroxide, hydroxyl radical and water. The electronic state for each activation step is shown with the energy of the reaction in Kcal/mole.

Hydrogen peroxide is noteworthy because it readily permeates membranes and it is therefore not compartmentalised in the cell. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecules. The well-known reactivity of hydrogen peroxide is not due to its reactivity per se, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical, which is the strongest oxidizing agent known and reacts with organic molecules at diffusion-limited rates.

Fenton described in the late nineteenth century (92, 93) the oxidising potential of hydrogen peroxide mixed with ferrous salts. Identified the hydroxyl radical as the oxidising species in these reactions:

(1) $$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

In biological systems the availability of ferrous ions limits the rate of reaction, but the recycling of iron from the ferric to the ferrous form by a reducing agent can maintain an ongoing Fenton reaction leading to the generation of hydroxyl radicals. One suitable reducing agent is superoxide which participates in the overall reaction 2 as two half reactions shown in reactions 3 and 4:

(2) $$\bullet O_2^- + H_2O_2 \rightarrow O_2 + \bullet OH + OH^-$$

(3) $$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

(4) $$\bullet O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

Therefore, in the presence of trace amounts of iron, the reaction of superoxide and hydrogen peroxide will form the destructive hydroxyl radical and initiate the oxidation of

organic substrates. Metals other than iron may also participate in these electron transfer reactions by cycling between oxidised and reduced states.

The oxidation of organic substances may proceed by two possible reactions Ä addition of OH to the organic molecule or abstraction of a hydrogen atom from it. In the addition reaction (reaction 5), the hydroxyl radical adds to an organic substrate forming a hydroxylated product that is further oxidised by ferrous ions, oxygen or other agents to a stable, oxidised product (reactions 6 and 7). The hydroxylated products can also dismutate to form cross-linked products (reaction 8).

(5) $\bullet OH + R \rightarrow \bullet ROH$

(6) $\bullet ROH + Fe^{3+} \rightarrow ROH + Fe^{2+} + H^{+}$

(7) $\bullet ROH + O_2 \rightarrow ROH + \bullet O_2^{-} + H^{+}$

(8) $\bullet ROH + \bullet ROH \rightarrow R - R + 2H_2O$

In the abstraction reaction, the hydroxyl radical oxidises the organic substrate forming water and an organic radical (reaction 9). The latter product has a single unpaired electron and thus can react with oxygen in the triplet ground-state (reaction 10). The addition of triplet oxygen to the carbon radical can lead to the formation of a peroxyl radical which can readily abstract hydrogen from another organic molecule leading to the formation of a second carbon radical (reaction 11). This chain reaction is why oxygen free radicals cause damage far in excess of their initial concentration.

(9) $\bullet OH + RH \rightarrow R\bullet + H_2O$

(10) $\bullet R + O_2 \rightarrow ROO\bullet$

(11) $ROO\bullet + RH \rightarrow R\bullet + ROOH$

BIOLOGICAL REACTIONS OF OXYGEN RADICALS:

The reactions of activated oxygen with organic substrates are complex even in vitro with homogenous solutions, but in biological systems there are even more complications due to the surface properties of membranes, electrical charges, binding properties of macromolecules, and compartmentalization of enzymes, substrates and catalysts. Thus, various sites even within a single cell differ in the nature and extent of reactions with oxygen.

The nature of the oxidative injury that causes cell death is not always obvious. The mechanisms by which oxygen radicals' damage membrane lipids are well accepted, and consequently oxidative damage is often exclusively associated with these peroxidation reactions in membrane lipids. What is sometimes overlooked in our research on environmental stress in plants is that activated forms of oxygen also degrade proteins and nucleic acids, reactions which can also be very lethal. In this section some of the major reactions of activated oxygen with lipids, protein, and nucleic acids are reviewed.

OXIDATIVE DAMAGE TO LIPIDS:

Classical Peroxidation Reactions:

The reactions of oxygen free radicals with polyunsaturated lipids have been extensively researched because of their involvement in rancidity and the development of undesirable odours and flavours in foods. Historically these reactions are the most frequently cited consequence of oxygen radical production in plant cells. Perhaps the mechanisms were so well established by oil chemists long before the recognition of their importance in biology that plant biologists applied these mechanisms directly to their experimental systems, rarely questioning their validity or transposability. This has delayed recognition of the presence of free radical reactions in plant membranes. The complexity of the biological membrane is well established and the reader is referred elsewhere for more detailed considerations of its structure (94). The lipid bilayer membrane is composed of a mixture of phospholipids and glycolipids that have fatty acid chains attached to carbon 1 and 2 of the glycerol backbone by an ester linkage. The peroxidation reactions differ among these fatty acids depending on the number and position of the double bonds on the acyl chain and the reader is a detailed review. The following is a simplified summary of these reactions for a general lipid, `R', and for a specific fatty acid, linoleate, which is common in plant cell membranes.

The peroxidation of lipids involves three distinct steps: initiation, propagation and termination. The initiation reaction between an unsaturated fatty acid (e.g. linoleate) and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid (reaction 9); in the case of linoleate this occurs at carbon-11 (Fig. 6). The remaining carbon centred radical, forms a resonance structure sharing this unpaired electron among carbons 9 to 13. In the propagation reactions, this resonance structure reacts with triplet oxygen, which remember is a biradical having two unpaired electrons and therefore reacts readily with other radicals. This reaction forms a peroxy radical (reaction 10). In the case of linoleate, addition occurs at either carbon-9 or -13 (Fig 6).

The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centred free radical (reaction 11) that can participate in a second H abstraction (reaction 10). Therefore, once one hydroxyl radical initiates the peroxidation reaction by abstracting a single H atom, it creates a carbon radical product (R) that is capable of reacting with ground state oxygen in a chain reaction. The role of the hydroxyl radical is analogous to a "spark" that starts a fire. The basis for the hydroxyl radical's extreme reactivity in lipid systems is that at very low concentrations it initiates a chain reaction involving triplet oxygen, the most abundant form of oxygen in the cell.

The lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalysts because ROOH will participate in a Fenton reaction leading to the formation of reactive alkoxy radicals:

(12) $$ROOH + Fe^{2+} \rightarrow OH^{-} + RO\bullet + Fe^{3+}$$

Therefore, in the presence of Fe, the chain reactions are not only propagated but amplified. Note that two radicals are produced by the summation of reactions 9 to 12. Among the degradation products of ROOH are aldehydes, such as malondialdehyde, and hydrocarbons, such as ethane and ethylene that are commonly measured end products of lipid peroxidation.

The peroxidation reactions in membrane lipids are terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals, such as those shown in reactions 13 to 15:

(13) $R\bullet + R\bullet \rightarrow R - R$

(14) $R\bullet + ROO\bullet \rightarrow ROOR$

(15) $ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$

Typically high molecular weight, cross-linked fatty acids and phospholipids accumulate in peroxidised membrane lipid samples.

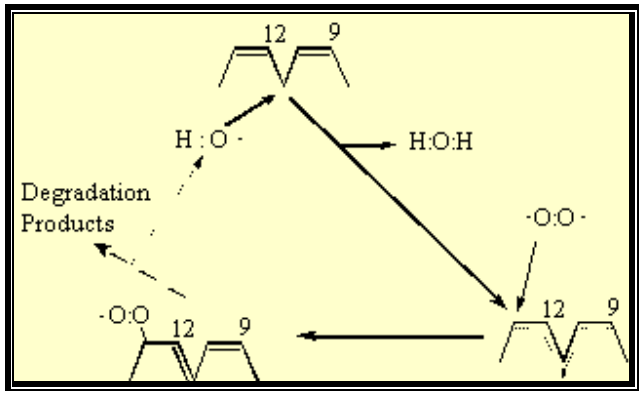

Figure 6: The peroxidation of linoleic acid. The hydroxyl radical abstracts a H atom from carbon-11 of the fatty acid between the two double bonds forming water. The electron deficiency is shared among carbons 9 to 13 in a resonance structure. Triplet oxygen that has two unpaired electrons may attach to this structure at either carbon -9 or -13 forming a peroxy radical. This peroxy radical will abstract another hydrogen atom from a second linoleic acid molecule in a propagation reaction forming a lipid hydroperoxide. Chain breakage and cross-linkage reactions subsequently occur to produce aldehydes, hydrocarbons, alcohols and cross-linked dimers.

Singlet oxygen can react readily with unsaturated fatty acids producing a complex mixture of hydroperoxides. Again, the chemistry of these reactions is based on foods. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products than the hydroxyl radical. Once formed the lipid hydroperoxides will decompose into a variety of products, some of which can produce oxygen free radicals in the presence of metal catalysts (reaction 12).

Unique Reactions in Plant Membranes:

The above mechanisms predict that oxygen free radical or lipid peroxidation reactions in plant membranes would selectively degrade unsaturated fatty acids and accumulate aldehydes, hydrocarbons, and cross-linked products.

OXIDATIVE DAMAGE TO PROTEINS:

Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The amino acids in a peptide differ in their susceptibility to attack, and the various forms of activated oxygen differ in their potential reactivity. Primary, secondary, and tertiary protein structures alter the relative susceptibility of certain amino acids. In spite of this complexity, generalisations can be made. Sulphur containing amino acids, and thiol groups specifically, are very susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Reduction of both of these may be accomplished in microbial systems by thioredoxin and thioredoxin reductase (95). A protein-methionine-S-oxide reductase has been measured in pea chloroplasts. This enzyme reduces the methionyl sulfoxide back to methionyl residues in the presence of thioredoxin. In some instances this enzyme has restored the biological activity of a protein, but this function in plants has not been described.

Other forms of free radical attack on proteins are not reversible. For example, the oxidation of iron-sulphur centres by superoxide destroys enzymatic function. Many amino acids undergo specific irreversible modifications when a protein is oxidised. For example, tryptophan is readily cross-linked to form bityrosine products (96). Histidine, lysine, proline, arginine, and serine form carbonyl groups on oxidation (97). The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as Fe. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidises an amino acid residue at or near the cation binding site of the protein (97). This site-specific alteration of an amino acid usually inactivates the enzyme by destruction of the cation binding site.

Oxidative modification of specific amino acids is one mechanism of marking a protein for proteolysis (97). In E. coli there are specific proteases that degrade oxidised proteins and similar specificity is expected in plants. It is well documented that the various peptide components of photosystem II turnover at different frequencies; the D1 protein specifically is noted for its high rate of turnover, and it is assumed that this is a consequence of oxidative attack at specific sites on the protein (98).

OXIDATIVE DAMAGE TO DNA:

Activated oxygen and agents that generate oxygen free radicals, such as ionising radiation, induce numerous lesions in DNA that cause deletions, mutations and other lethal genetic effects. Characterisation of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing base degradation, single strand breakage, and cross-linking to protein (99). Degradation of the base will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and -saturated products.

The principle cause of single strand breaks is oxidation of the sugar moiety by the hydroxyl radical. In vitro neither hydrogen peroxide alone nor superoxide cause strand breaks under physiological conditions, and therefore, their toxicity in vivo is most likely the result of Fenton reactions with a metal catalyst. At least in E. coli NADH can drive these Fenton reactions. For example, the ndh mutant in E. coli accumulates NADH as a result of the mutant's

inability to donate electrons from NADH to respiratory pathways; as a result, the mutant is hypersensitive to hydrogen peroxide. Studies of other E. coli mutants have lead to the conclusion that a Fenton active metal is bound to DNA, probably chelated to phosphodiester linkage. If the bound metal is reduced by a small diffusible molecule, such as NAD(P)H or superoxide, it will react with hydrogen peroxide to form the hydroxyl radical. The short-lived hydroxyl radical then oxidises an adjacent sugar or base causing breakage of the DNA chain.

Cross-linking of DNA to protein is another consequence of hydroxyl radical attack on either DNA or its associated proteins (100). Treatment with ionising radiation or other hydroxyl radical generating agents causes covalent leakages such as thymine cysteine addicts, between DNA and protein. When these cross-linkages exist, separation of protein from DNA by various extraction methods is ineffective. Although DNA-protein cross-links are about an order of magnitude less abundant than single strand breaks, they are not as readily repaired, and may be lethal if replication or transcription precedes repair.

DNA is an obvious weak link in a cell's ability to tolerate oxygen free radical attack. First, it seems that DNA is effective in binding metals that are involved in Fenton reactions, and secondly less damage can be tolerated in DNA than other macromolecules. As a consequence, the cell has a number of DNA repair enzymes. One reason why eukaryotic organisms have compartmentalised DNA in the nucleus, away from sites of redox cycling that are high in NAD (P) H and other reductants, may be to avoid oxidative damage (101).

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