

## **DIABETES AND OXIDATIVE STRESS- A REVIEW**

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## WHAT IS DIABETES ?

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin, or alternatively, when the body cannot effectively use the insulin it produces. Insulin is a hormone that regulates blood sugar. Hyperglycaemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels (1)

### CLASSIFICATION OF DIABETES MELLITUS

Based on etiology diabetes mellitus is classified as follow

- I Type 1 diabetes** (previously known as insulin-dependent or childhood-onset) is characterized by a lack of insulin production. Without daily administration of insulin, Type 1 diabetes is rapidly fatal.
  - a. Immune mediated
  - b. Idiopathic
  - ✚ Symptoms include excessive excretion of urine (polyuria), thirst (polydipsia), constant hunger, weight loss, vision changes and fatigue. These symptoms may occur suddenly.
- II Type 2 diabetes** (formerly called non-insulin-dependent or adult-onset) results from the body's ineffective use of insulin. Type 2 diabetes comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and physical inactivity.
  - ✚ Symptoms may be similar to those of Type 1 diabetes, but are often less marked. As a result, the disease may be diagnosed several years after onset, once complications have already arisen.
  - ✚ Until recently, this type of diabetes was seen only in adults but it is now also occurring in obese children.
- III Gestational diabetes** is hyperglycaemia which is first recognized during pregnancy.
  - ✚ Symptoms of gestational diabetes are similar to Type 2 diabetes. Gestational diabetes is most often diagnosed through prenatal screening, rather than reported symptoms.
- IV Other specific types**
  1. Genetic defects of  $\beta$ -cell function
  2. Genetic defects in insulin action
  3. Diseases of the exocrine pancreas
  4. Endocrinopathies
  5. Drug- or chemical-induced
  6. Infections
  7. Uncommon forms of immune-mediated diabetes
  8. Other genetic syndromes sometimes associated with diabetes

Impaired Glucose Tolerance (IGT) and Impaired Fasting Glycaemia (IFG) are intermediate conditions in the transition between normality and diabetes. People with IGT or IFG are at high risk of progressing to type 2 diabetes, although this is not inevitable.

## DIABETES FACTS

- ✓ The World Health Organization (WHO) estimates that more than 180 million people worldwide have diabetes. This number is likely to more than double by 2030.
- ✓ In 2005, an estimated 1.1 million people died from diabetes.<sup>1</sup>
- ✓ Almost 80% of diabetes deaths occur in low and middle-income countries.
- ✓ Almost half of diabetes deaths occur in people under the age of 70 years; 55% of diabetes deaths are in women.
- ✓ WHO projects that diabetes death will increase by more than 50% in the next 10 years without urgent action. Most notably, diabetes deaths are projected to increase by over 80% in upper-middle income countries between 2006 and 2015.

## WHAT ARE COMMON CONSEQUENCES OF DIABETES?

Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, and nerves.

- **Diabetic retinopathy** is an important cause of blindness, and occurs as a result of long-term accumulated damage to the small blood vessels in the retina. After 15 years of diabetes, approximately 2% of people become blind, and about 10% develop severe visual impairment.
- **Diabetic neuropathy** is damage to the nerves as a result of diabetes, and affects up to 50% of people with diabetes. Although many different problems can occur as a result of diabetic neuropathy, common symptoms are tingling, pain, numbness, or weakness in the feet and hands.
- Combined with reduced blood flow, neuropathy in the feet increases the chance of **foot ulcers** and eventual **limb amputation**.
- Diabetes is among the leading causes of kidney failure. 10-20% of people with diabetes die of **kidney failure**.
- Diabetes increases the risk of **heart disease and stroke**. 50% of people with diabetes die of cardiovascular disease (primarily heart disease and stroke).
- The overall risk of dying among people with diabetes is at least double the risk of their peers without diabetes.

## WHAT IS OXIDATIVE STRESS ?

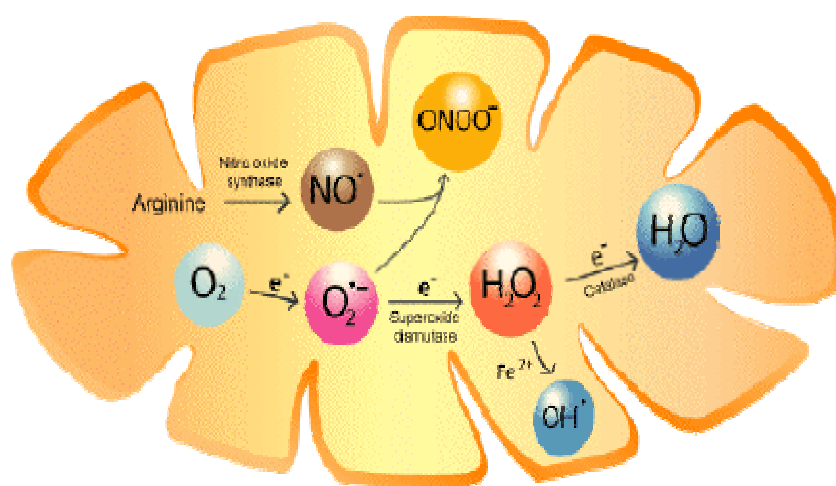
Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. All forms of life maintain a reducing environment within their cells.

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.

In humans, oxidative stress is involved in many diseases, such as atherosclerosis, Parkinson's disease, Heart Failure, Myocardial Infarction, Alzheimer's disease and chronic fatigue syndrome, but short-term oxidative stress may also be important in prevention of aging by induction of a process named mitohormesis (2). Reactive oxygen species can be beneficial, as they are used by the immune system as a way to attack and kill pathogens. Reactive oxygen species are also used in cell signaling. This is dubbed redox signaling.

Mitochondria are a primary site of production of free radicals. While more than 98% of the molecular oxygen taken up by cells is fully utilized by cytochrome c oxidase to form water, this enzyme can release partly reduced species. Other enzymes of the respiratory chain, and in particular complexes I and III, also produce partly reduced oxygen species including superoxide.

These reactive oxygen species can react with nitric oxide to produce reactive nitrogen species including peroxynitrite. While a significant proportion of the reactive oxygen and nitrogen species diffuse into the cytosol, a major portion remains in the mitochondrion. The level of reactive oxygen and nitrogen species produced is a function of the activity and more specifically the dysfunction of the OXPHOS chain. The less efficient electron transfer is, the more radicals are produced. Free radical damage of cytosolic proteins increases with increased levels of radical production. Moreover, oxidative and nitrative damage of mitochondrial proteins adds to OXPHOS dysfunction further exacerbating free radical production.



## CHEMICAL AND BIOLOGICAL EFFECTS

In chemical terms, oxidative stress is a large rise (becoming less negative) in the cellular reduction potential, or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione (3). The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis (4).

A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage (5). The major portion of long term effects is inflicted by damage on DNA (6). Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism and the damage they cause to cells is constantly repaired. However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (7-8).

Oxidant	Description
$\bullet\text{O}_2^-$ , superoxide anion	One-electron reduction state of $\text{O}_2$ , formed in many autoxidation reactions and by the electron transport chain. Rather unreactive but can release $\text{Fe}^{2+}$ from iron-sulfur proteins and ferritin. Undergoes dismutation to form $\text{H}_2\text{O}_2$ spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed $\bullet\text{OH}$ formation.
$\text{H}_2\text{O}_2$ , hydrogen peroxide	Two-electron reduction state, formed by dismutation of $\bullet\text{O}_2^-$ or by direct reduction of $\text{O}_2$ . Lipid soluble and thus able to diffuse across membranes.
$\bullet\text{OH}$ , hydroxyl radical	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxyxynitrite. Extremely reactive, will attack most cellular components
$\text{ROOH}$ , organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and nucleobases.
$\text{RO}\bullet$ , alkoxy and $\text{ROO}\bullet$ , peroxy radicals	Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.

HOCl, hypochlorous acid	Formed from H <sub>2</sub> O <sub>2</sub> by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
ONOO <sup>-</sup> , peroxynitrite	Formed in a rapid reaction between •O <sub>2</sub> <sup>-</sup> and NO•. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.

Table adapted from (9-11).

## PRODUCTION AND CONSUMPTION OF OXIDANTS

One source of reactive oxygen under normal conditions in humans is the leakage of activated oxygen from mitochondria during oxidative phosphorylation. However, *E. coli* mutants that lack an active electron transport chain produced as much hydrogen peroxide as wild-type cells, indicating that other enzymes contribute the bulk of oxidants in these organisms (12). One possibility is that multiple redox-active flavoproteins all contribute a small portion to the overall production of oxidants under normal conditions (13-14).

Other enzymes capable of producing superoxide are xanthine oxidase, NADPH oxidases and cytochromes P450. Hydrogen peroxide is produced by a wide variety of enzymes including several oxidases. Reactive oxygen species play important roles in cell signalling, a process termed redox signaling. Thus, to maintain proper cellular homeostasis, a balance must be struck between reactive oxygen production and consumption.

The best studied cellular antioxidants are the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase. Less well studied (but probably just as important) enzymatic antioxidants are the peroxiredoxins and the recently discovered sulfiredoxin. Other enzymes that have antioxidant properties (though this is not their primary role) include paraoxonase, glutathione-S transferases, and aldehyde dehydrogenases.

Oxidative stress contributes to tissue injury following irradiation and hyperoxia. It is suspected (though not proven) to be important in neurodegenerative diseases including Lou Gehrig's disease (aka MND or ALS), Parkinson's disease, Alzheimer's disease, and Huntington's disease. Oxidative stress is thought to be linked to certain cardiovascular disease, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation. Oxidative stress also plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes both strokes and heart attacks. Oxidative stress has also been implicated in chronic fatigue syndrome (15).

### **ANTIOXIDANTS AS SUPPLEMENTS**

The use of antioxidants to prevent disease is controversial (16). In a high-risk group like smokers, high doses of beta carotene increased the rate of lung cancer (17). In less high-risk groups, the use of vitamin E appears to reduce the risk of heart disease (18). In other diseases, such as Alzheimer's, the evidence on vitamin E supplementation is mixed (19, 20). However, AstraZeneca's radical scavenging nitron drug NXY-059 shows some efficacy in the treatment of stroke (21).

Oxidative stress (as formulated in Harman's free radical theory of aging) is also thought to contribute to the aging process. While there is good evidence to support this idea in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, (22, 23)] recent evidence from Michael Ristow's laboratory suggests that oxidative stress may also promote life expectancy of *Caenorhabditis elegans* by inducing a secondary response to initially increased levels of reactive oxygen species (24). This process was previously named mitohormesis or mitochondrial hormesis on a purely hypothetical basis (25). The situation in mammals is even less clear (26-28). Recent epidemiological findings support the process of mitohormesis, and even suggest that antioxidants may increase disease prevalence in humans (although the results were influenced by studies on smokers) (29).

### **METAL CATALYSTS**

Metals such as iron, copper, chromium, vanadium and cobalt are capable of redox cycling in which a single electron may be accepted or donated by the metal. This action catalyzes reactions that produce reactive radicals and can produce reactive oxygen species. The most important reactions are probably Fenton's reaction and the Haber-Weiss reaction, in which hydroxyl radical is produced from reduced iron and hydrogen peroxide. The hydroxyl radical then can lead to modifications of amino acids (e.g. meta-tyrosine and ortho-tyrosine formation from phenylalanine), carbohydrates, initiate lipid peroxidation, and oxidize nucleobases. Most enzymes that produce reactive oxygen species contain one of these metals. The presence of such metals in biological systems in an uncomplexed form (not in a protein or other protective metal complex) can significantly increase the level of oxidative stress. In humans, hemochromatosis is associated with increased tissue iron levels, Wilson's disease with increased tissue levels of copper and chronic manganism with exposure to manganese ores.

### **NON-METAL REDOX CATALYSTS**

Certain organic compounds in addition to metal redox catalysts can also produce reactive oxygen species. One of the most important classes of these are the quinones. Quinones can redox cycle with their conjugate semiquinones and hydroquinones, in some cases catalyzing the production of superoxide from dioxygen or hydrogen peroxide from superoxide.

Oxidative stress generated by the reducing agent uric acid may be involved in the Lesch-Nyhan syndrome, stroke, and metabolic syndrome. Likewise, production of reactive oxygen species in the presence of homocysteine may figure in homocystinuria, as well as atherosclerosis, stroke, and Alzheimers.

### **IMMUNE DEFENSE**

The immune system uses the lethal effects of oxidants by making production of oxidizing species a central part of its mechanism of killing pathogens; with activated phagocytes producing both ROS and reactive nitrogen species. These include superoxide ( $\bullet\text{O}_2^-$ ), nitric oxide ( $\bullet\text{NO}$ ) and their particularly reactive product, peroxynitrite ( $\text{ONOO}^-$ ) (30). Although the use of these highly reactive compounds in the cytotoxic response of phagocytes causes damage to host tissues, the non-specificity of these oxidants is an advantage since they will damage almost every part of their target cell (11). This prevents a pathogen from escaping this part of immune response by mutation of a single molecular target.

### **REACTIVE OXYGEN SPECIES**

Reactive oxygen species (ROS) are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress (such as for example, UV or heat exposure) ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. They are also generated by exogenous sources such as ionizing radiation.

### **DAMAGING EFFECTS**

Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases, catalases, glutathione peroxidases and peroxiredoxins. Small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, and glutathione also play important roles as cellular antioxidants. Similarly, polyphenol antioxidants assist in preventing ROS damage by scavenging free radicals. In contrast, the antioxidant ability of the extracellular space is relatively less--e.g., the most important plasma antioxidant in humans is probably uric acid.

Effects of ROS on cell metabolism have been well documented in a variety of species. These include not only roles in apoptosis (programmed cell death), but also positive effects such as the induction of host defence genes and mobilisation of ion transport systems. This is implicating them more frequently with roles in redox signaling or oxidative signaling.



In particular, platelets involved in wound repair and blood homeostasis release ROS to recruit additional platelets to sites of injury. These also provide a link to the adaptive immune system via the recruitment of leukocytes.

Reactive oxygen species are implicated in cellular activity to a variety of inflammatory responses including cardiovascular disease. They may also be involved in hearing impairment via cochlear damage induced by elevated sound levels, ototoxicity of drugs such as cisplatin, and in congenital deafness in both animals and humans. Redox signaling is also implicated in mediation of apoptosis or programmed cell death and ischaemic injury. Specific examples include stroke and heart attack.

Generally, harmful effects of reactive oxygen species on the cell are most often:

1. Damage of DNA
2. Oxidations of polydesaturated fatty acids in lipids
3. Oxidations of amino acids in proteins
4. Oxidatively inactivate specific enzymes by oxidation of co-factors

## **OXIDATIVE DAMAGE**

In aerobic organisms the energy needed to fuel biological functions is produced in the mitochondria via the electron transport chain. In addition to energy, reactive oxygen species (ROS) are produced which have the potential to cause cellular damage. ROS can damage DNA, RNA, and proteins which theoretically contributes to the physiology of ageing.

ROS are produced as a normal product of cellular metabolism. In particular, one major contributor to oxidative damage is hydrogen peroxide ( $H_2O_2$ ) which is converted from superoxide that leaks from the mitochondria. Within the cell there is catalase and superoxide dismutase that help to minimize the damaging effects of hydrogen peroxide by converting it into oxygen and water, benign molecules, however this conversion is not 100% efficient, and residual peroxides persist in the cell. While ROS are produced as a product of normal cellular functioning, excessive amounts can cause deleterious effects (31).

Memory capabilities decline with age, evident in human degenerative diseases such as Alzheimer's disease which is accompanied by an accumulation of oxidative damage. Current studies demonstrate that the accumulation of ROS can decrease an organism's fitness because oxidative damage is a contributor to senescence. In particular, the accumulation of oxidative damage may lead to cognitive dysfunction as demonstrated in a study where old rats were given mitochondrial metabolites and then given cognitive tests, results showed that the rats performed better after receiving the metabolites, suggesting that the metabolites reduced oxidative damage and improved mitochondrial function (32). Accumulating oxidative damage can then affect the efficiency of mitochondria and further increase the rate of ROS production (33).

The accumulation of oxidative damage and its implications for aging depends on the particular tissue type where the damage is occurring. Additional experimental results suggest that oxidative damage is responsible for age related decline in brain functioning. Older gerbils were found to have higher levels of oxidized protein in comparison to younger gerbils. When old and young mice were treated with a spin trapping compound the level of oxidized proteins decreased in older gerbils but did not have an effect on younger gerbils. Additionally, older gerbils performed cognitive tasks better during treatment but ceased functional capacity when treatment was discontinued causing oxidized protein levels to increase. This lead researcher to conclude that oxidation of cellular proteins is potentially important for brain function.

### **INTERNAL PRODUCTION**

Free radicals are also produced inside (and also released towards the cytosol (34, 35). organelles, such as the mitochondrion. Mitochondria convert energy for the cell into a usable form, adenosine triphosphate (ATP). The process in which ATP is produced, called oxidative phosphorylation, involves the transport of protons (hydrogen ions) across the inner mitochondrial membrane by means of the electron transport chain. In the electron transport chain, electrons are passed through a series of proteins via oxidation-reduction reactions, with each acceptor protein along the chain having a greater reduction potential than the last. The last destination for an electron along this chain is an oxygen molecule. Normally the oxygen is reduced to produce water; however, in about 0.1-2% of electrons passing through the chain (this number derives from studies in isolated mitochondria, though the exact rate in live organisms is yet to be fully agreed upon), oxygen is instead prematurely and incompletely reduced to give the superoxide radical,  $\bullet\text{O}_2^-$ , most well documented for Complex I and Complex III. Superoxide is not particularly reactive in and of itself, but can inactivate specific enzymes or initiate lipid peroxidation in its  $\text{HO}_2\bullet$  form. If too much damage is caused to its mitochondria, a cell undergoes apoptosis or programmed cell death.

Bcl-2 proteins are layered on the surface of the mitochondria, detect damage, and activate a class of proteins called Bax, which punch holes in the mitochondrial membrane, causing cytochrome C to leak out. This cytochrome C binds to Apaf-1, or apoptotic protease activating factor-1, which is free-floating in the cell's cytoplasm. Using energy from the ATPs in the mitochondrion, the Apaf-1 and cytochrome C bind together to form apoptosomes. The apoptosomes binds to and activates caspase-9, another free-floating protein. The caspase-9 then cleaves the proteins of the mitochondrial membrane, causing it to break down and start a chain reaction of protein denaturation and eventually phagocytosis of the cell.

### **CAUSE OF AGING**

According to the Free-radical theory, oxidative damage initiated by reactive oxygen species is a major contributor to the functional decline that is characteristic of aging.

While studies in invertebrate models indicate that animals genetically engineered to lack specific antioxidant enzymes (such as SOD) generally show a shortened lifespan (as one would expect from the theory), the converse, increasing the levels of antioxidant enzymes, has yielded inconsistent effects on lifespan (though some well-performed studies in *Drosophila* do show that lifespan can be increased by the overexpression of MnSOD or glutathione biosynthesizing enzymes). In mice, the story is somewhat similar. Deleting antioxidant enzymes generally yields shorter lifespan, though overexpression studies have not (with some recent exceptions), consistently extended lifespan (36).

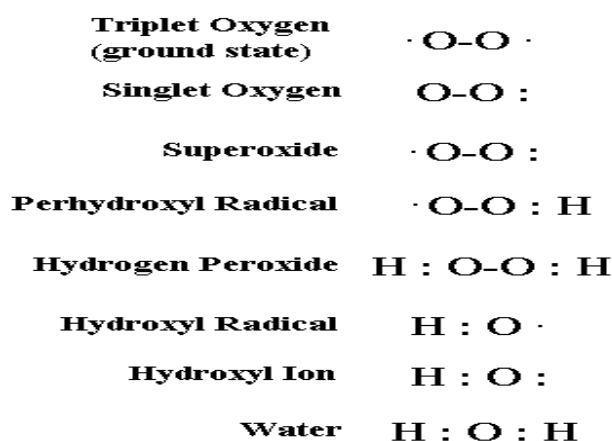
### **ACTIVATION OF OXYGEN:**

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. 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:

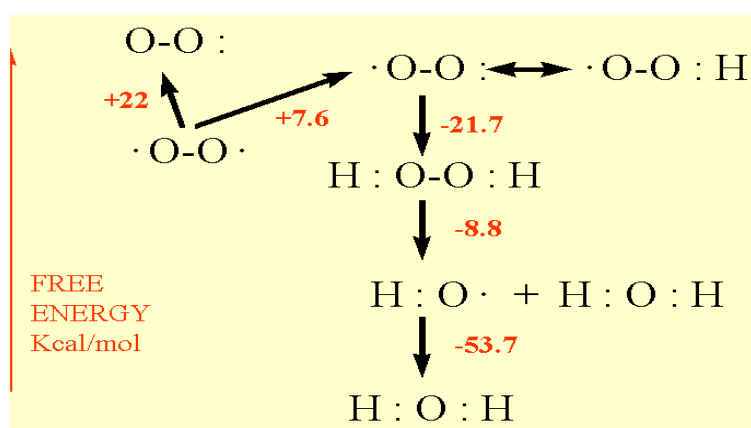
Absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins (Fig. 1).

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 1:** Nomenclature of the various forms of oxygen

The second mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl radical ( $\text{OH}$ ) 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.



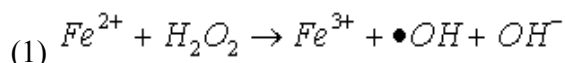
**Figure 2:** The activation states of oxygen. Non-activated oxygen is a biradical. From this triplet state it can be activated by either reversing the spin on one of the unpaired electrons to form the singlet state or by reduction. The first reduction reaction is endothermic forming superoxide. Subsequent 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.

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, but its biological relevance is probably minor because of its low concentration at physiological pH.

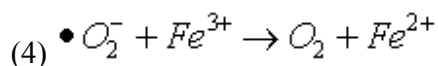
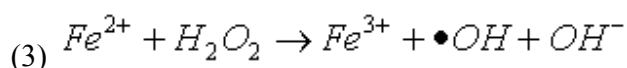
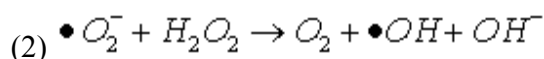
The univalent reduction of superoxide produces hydrogen peroxide that is not a free radical because all of its electrons are paired (Fig. 2). Very often the reduction products of oxygen are referred to by biologists as oxygen free radicals which is a misnomer because in chemistry a free radical is defined 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.

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 the oxidising potential of hydrogen peroxide mixed with ferrous salts. Identified the hydroxyl radical as the oxidising species in these reactions:

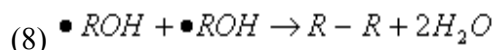
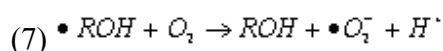
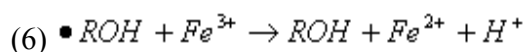
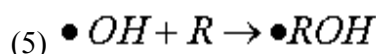


In biological systems the availability of ferrous ions limits the rate of reaction, but the recycling of iron from the ferric to the ferrous form by a reducing agent can maintain an ongoing Fenton reaction leading to the generation of hydroxyl radicals. One suitable reducing agent is superoxide which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

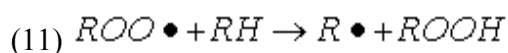
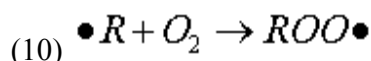
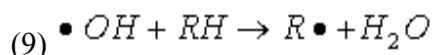


Therefore, in the presence of trace amounts of iron, the reaction of superoxide and hydrogen peroxide will form the destructive hydroxyl radical and initiate the oxidation of organic substrates. Metals other than iron may also participate in these electron transfer reactions by cycling between oxidised and reduced states.

The oxidation of organic substances may proceed by two possible reactions. An 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).



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 peroxy 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.



### **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:**

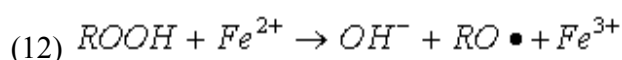
The reactions of oxygen free radicals with polyunsaturated lipids have been extensively researched because of their involvement in rancidity and the development of undesirable odours and flavours in foods. Historically these reactions are the most frequently cited consequence of oxygen radical production 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. 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. 3). 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 3).

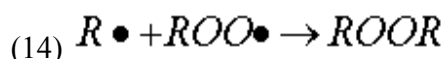
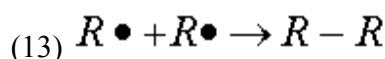
The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centred free radical (reaction 11) that can participate in a second H abstraction (reaction 10). Therefore, once one hydroxyl radical initiates the peroxidation reaction by abstracting 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.

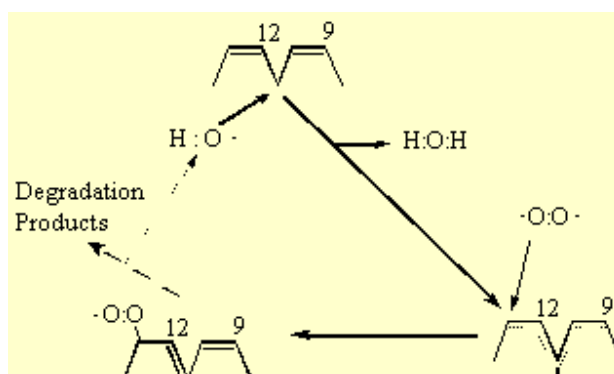
The lipid hydroperoxide (ROOH) is unstable in the presence of Fe or other metal catalysts because ROOH will participate in a Fenton reaction leading to the formation of reactive alkoxy radicals:



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:



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



**Figure 3:** 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. 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. Histidine, lysine, proline, arginine, and serine form carbonyl groups on oxidation. 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. 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. 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.

### **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. 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 led 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. Treatment with ionising radiation or other hydroxyl radical generating agents causes covalent leakages such as thymine-cysteine adducts, 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.

## SITES OF ACTIVATED OXYGEN PRODUCTION:

As indicated above, there are two forms of activated oxygen that are formed by distinctly different mechanisms. The reduction of oxygen to form superoxide, hydrogen peroxide and hydroxyl radicals is the principle mechanism of oxygen activation in most biological systems. However in photosynthetic plants, the formation of singlet oxygen by the photosystems has importance. Activated oxygen is often formed as a component of metabolism to enable "complex" chemical reactions, such as the oxidation of xenobiotics or the polymerisation of lignin, but in other instances activated oxygen is formed by the dysfunctioning of enzymes or electron transport systems, as a result of perturbations in metabolism caused by chemical or environmental stress.

### CHLOROPLASTS:

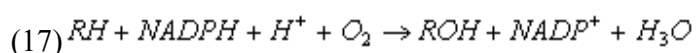
There are at least four sites within the chloroplast that can activate oxygen.

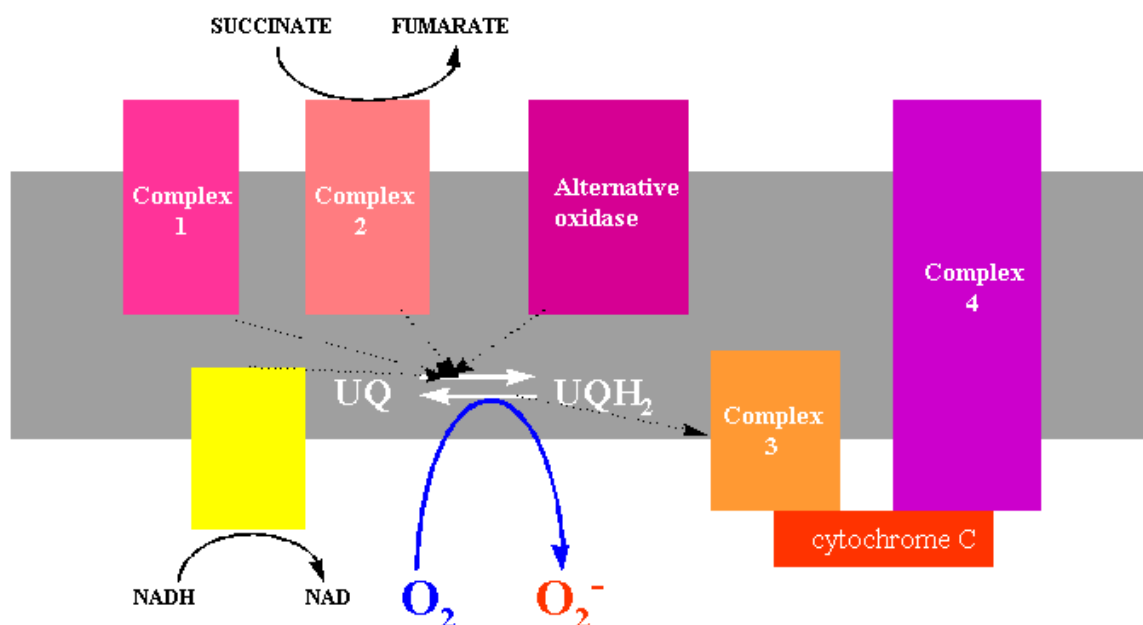
### MITOCHONDRIA:

Most oxygen is consumed by the cytochrome oxidase enzyme in the mitochondrial electron transport system, and involves the sequential transfer of four electrons to oxygen, releasing water. Plant mitochondria have an additional site of oxygen reduction at the alternative oxidase, distinguished from cytochrome oxidase by its resistance to cyanide. However, neither of these sites produces significant quantities of superoxide. However, isolated mitochondria produce  $H_2O_2$  and  $O_2$  in the presence of NADH. Antimycin A which blocks electron flow after ubiquinone (Fig. 6) enhances oxygen reduction. Presumably other conditions which also increase the reduction of ubiquinone favour reduction of oxygen in the ubiquinone  $\ddot{A}$  cytochrome b region of the chain. The various Fe-S proteins and NADH dehydrogenase have also been implicated as possible sites of superoxide and hydrogen peroxide formation.

### ENDOPLASMIC RETICULUM:

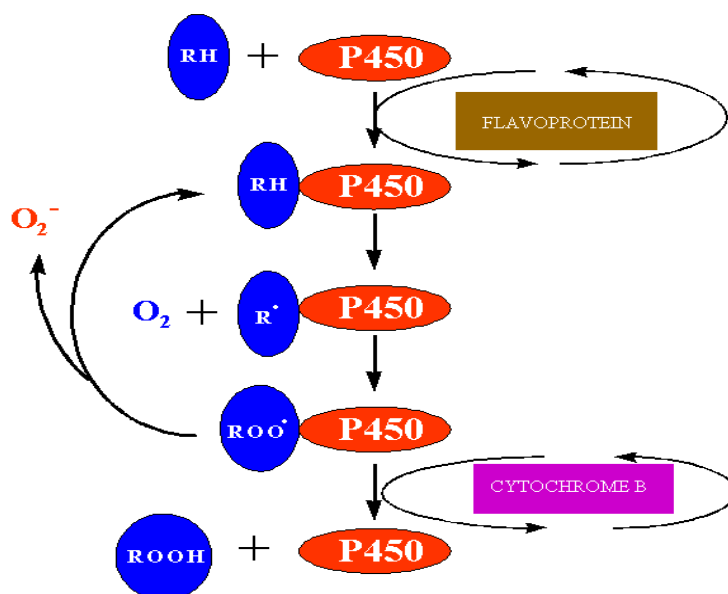
Various oxidative processes, including oxidation, hydroxylations, dealkylations, deaminations, dehalogenation and desaturation, occur on the smooth endoplasmic reticulum. Mixed function oxygenases that contain a heme moiety add an oxygen atom into an organic substrate using NAD(P)H as the electron donor. The generalised reaction catalysed by cytochrome  $P_{450}$  is:





**Figure 4:** Schematic representation of the electron transport system in the mitochondrial membrane showing a possible site of superoxide production by reduced ubiquinones.

The best characterised cytochrome P450 in plants is cinnamate-4-hydroxylase which functions in flavonoid and lignin biosynthesis, but other mixed function oxidases function in other biochemical pathways including gibberellin and sterol biosynthesis. Activation of oxygen by these systems is an essential prerequisite to oxygen addition reactions in the synthesis of these "complex" metabolites. Superoxide is produced by microsomal NAD (P) H dependent electron transport involving cytochrome P450. One possible site at which this may occur is shown in figure 7. After the univalent reduction of the substrate (RH) and the addition of triplet oxygen to form the complex P450 - RHOO the complex may decompose to P450-RH and release superoxide.



**Figure 5. Schematic representation of the cytochrome P<sub>450</sub> electron transport system on the endoplasmic reticulum showing one possible site of superoxide production.**

Cytochrome P450 reacts first with its organic substrate, RH. The complex is reduced by a flavoprotein to form a radical intermediate that can readily react with triplet oxygen because each has one unpaired electron. This oxygenated complex may be reduced by cytochrome b or occasionally the complex may decompose releasing superoxide.

### MICROBODIES:

Peroxisomes and glyoxysomes are organelles with a single membrane that compartmentalises enzymes involved in the  $\beta$ -oxidation of fatty acids, and the glyoxylic acid cycle including glycolate oxidase, catalase and various peroxidases. Glycolate oxidase produces  $H_2O_2$  in a two electron transfer from glycolate to oxygen. Xanthine oxidase, urate oxidase and NADH oxidase generate superoxide as a consequence of the oxidation of their substrates. The xanthine oxidase reaction is often used in vitro as a source of superoxide producing one mole of superoxide during the conversion of xanthine to uric acid.

### PLASMA MEMBRANES:

A superoxide-generating NAD(P)H oxidase activity has been clearly identified in plasmalemma enriched fractions. These flavoproteins may produce superoxide by the redox cycling of certain quinones or nitrogenous compounds. In the root, NAD (P)H oxidase reduces  $Fe^{3+}$  to  $Fe^{2+}$  converting it to a form that can be transported. Dysfunction of this root enzyme will produce superoxide. An auxin-activated NADH oxidase has been associated with acidification of the cell wall and auxin-stimulated cell elongation.

The plant NAD(P)H oxidase may have an analogous function to the animal enzyme. Leucocytes contain an NADH oxidase on the outer membrane surface which is activated in response to a foreign agent, generating superoxide that initiates oxidative reactions that destroy the potential pathogen. In plants, fungal elicitors cause a similar formation of superoxide that has been linked to the hypersensitive response to some pathogenic fungi. Wounding, heat shock and xenobiotics transiently activate this superoxide generating reaction, and consequently, it has been proposed that these superoxide generating reactions may serve as a signal in plant cells to elicit responses to biological, physical or chemical stress.

### CELL WALLS:

Although it is not immediately obvious, cell walls are active sites of metabolism, and also oxygen activation. Some of these reactions may be involved in the defense reactions against pathogens as described above. Others may involve the degradation or compartmentation of xenobiotic chemicals. However, the most common reactions are biosynthetic. For example, the phenylpropanoid precursors of lignin are crosslinked by H<sub>2</sub>O<sub>2</sub> dependent reactions that randomly link the subunits to form lignin. NADH is generated by a cell wall malate dehydrogenase, and then used to form H<sub>2</sub>O<sub>2</sub>, possibly by the NADH oxidase on the plasmalemma. Diamine oxidases are also involved in production of activated oxygen in the cell wall using diamines or polyamines (putrescine, spermidine, cadaverine, etc.) to reduce a quinone that will autoxidize, forming peroxides.

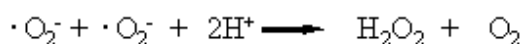
### DEFENCE MECHANISMS:

#### SUPEROXIDE DISMUTASE:

Superoxide dismutase (SOD) was be a copper storage protein. Subsequently, the enzyme was identified by a number of names, erythrocuprein, indophenol oxidase, and tetrazolium oxidase until its catalytic function. SOD is now known to catalyse the dismutation of superoxide to hydrogen peroxide and oxygen:

Therefore, the activity of this enzyme determines the relative proportions of the two constituents of the Haber-Weiss reaction that generates hydroxyl radicals (reaction 2).

Superoxide Dismutase



Mn-SOD: mitochondria

Fe-SOD: chloroplast

CuZn-SOD: chloroplast

CuZn-SOD: cytosol

(18)

Since SOD is present in all aerobic organisms and most (if not all) subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defence against oxidative stress. There are three distinct types of SOD classified on the basis of the metal cofactor: the copper/zinc (Cu/Zn - SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes. These isozymes can be separated by native polyacrylamide gel electrophoresis, their activity detected by negative staining and identified on the basis of their sensitivity to KCN and H<sub>2</sub>O<sub>2</sub>. The Mn-SOD is resistant to both inhibitors, whereas the Cu/Zn-SOD is sensitive to both inhibitors; Fe-SOD is resistant to KCN, and sensitive to H<sub>2</sub>O<sub>2</sub>. The subcellular distribution of these isozymes is also distinctive. The Mn-SOD is found in the mitochondria of eukaryotic cells; some Cu/Zn-SOD isozymes are found in the cytosol, others in the chloroplasts of higher plants. The Fe-SOD isozymes are often not detected in plants, but when detected, Fe-SOD is usually associated with the chloroplast compartment. The prokaryotic Mn-SOD and Fe-SOD, and the eukaryotic Cu/Zn-SOD enzymes are dimers, whereas the Mn-SOD of mitochondria is tetramers. Peroxisomes and glyoxysomes of watermelons (*Citrullus vulgaris*) have been shown to contain both Cu/Zn- and Mn-SOD activity, but there are no reports of extracellular SOD enzymes in plants. All forms of the SOD are nuclear-encoded and are targeted to their respective subcellular compartments by an amino terminal targeting sequence. Several forms of SOD have been cloned from a variety of plants.

Prokaryotic cells, and many eukaryotic algae contain only the Mn-SOD and Fe-SOD isozymes which are believed to be more ancient forms. In the bacteria *E. coli*, SOD activity is transcriptionally regulated by the SOX RS operon but investigations into the regulatory mechanism of SOD expression in plants are only beginning. To date it has been shown that SOD activity is increased in cells in response to diverse environmental and xenobiotic stresses including paraquat, high light, waterlogging and drought. Apparently, each of the SOD isozymes are independently regulated according to the degree of oxidative stress experienced in the respective subcellular compartments, but how this is communicated at the molecular level is unknown. In all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (microbodies) by oxidases involved in  $\beta$ -oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purine catabolism. Catalase was one of the first enzymes to be isolated in a highly purified state. All forms of the enzyme are tetramers in excess of 2, 20,000 molecular weight. Multiple forms of catalase have been described in many plants. These forms have been cloned from maize and homologous genes have been cloned from several other plants. Maize has three isoforms termed cat-1, cat-2 and cat-3, which are on separate chromosomes and are differentially expressed and independently regulated. Cat-1 and cat-2 are localised in peroxisomes and the cytosol, whereas cat-3 is mitochondrial. Careful examination of the structure of beef liver catalase has shown four NADPH binding sites per catalase tetramer but these sites were not in close association with the hydrogen peroxide reaction centre. Instead, NADPH functions in animal catalase to protect against inactivation by hydrogen peroxide.

The only plant catalase examined, potato, does not contain NADPH. It is interesting in this regard to note that catalase is very sensitive to light and has a rapid turnover rate similar to that of the D1 protein of PSII. This may be a result of light absorption by the heme group or perhaps hydrogen peroxide inactivation. Regardless, stress conditions that reduce the rate of protein turnover, such as salinity, heat shock or cold, cause the depletion of catalase activity. This may have significance in the plant's ability to tolerate the oxidative components of these environmental stresses.

### **GLUTATHIONE:**

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly) whose antioxidant function is facilitated by the sulphhydryl group of cysteine. On oxidation, the sulphur forms a thiyl radical that reacts with a second oxidised glutathione forming a disulphide bond (GSSG). Some legumes contain homoglutathione (hGSH) that is a homologous tripeptide of Glu-Cys-Ala. Detailed reviews of GSH chemistry are available elsewhere. GSH has a redox potential of -340 mV that enables GSH to reduce dehydroascorbate to ascorbate or to reduce the disulphide bonds of proteins.

GSH is found in most tissues, cells and subcellular compartments of higher plants. Its levels declines with tissue age and vary among growth environments; for example, glutathione levels are higher in the light than in the dark. At the subcellular level, GSH concentration is highest in the chloroplast, averaging between 1 and 4 mM, but significant quantities also accumulate in the cytosol. GSH exists predominantly in the reduced form with estimates varying from 70% in barley chloroplasts to 90% in pea chloroplasts.

GSH can function as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger. GSH may stabilise membrane structure by removing acyl peroxides formed by lipid peroxidation reactions. As detailed in section 2.4.3, GSH is the reducing agent that recycles ascorbic acid from its oxidised to its reduced form by the enzyme dehydroascorbate reductase. GSH can also reduce dehydroascorbate by a non-enzymatic mechanism at pH > 7 and GSH concentrations greater than 1 mM. This may be a significant pathway in chloroplasts whose stromal pH in the light is about 8 and GSH concentrations may be as high as 5 mM.

There are alternative functions for GSH in cellular metabolism independent of its antioxidant properties. It may have a significant role in the transport of reduced sulphur from leaves to sink tissues such as the root. GSH also participates in the detoxification of xenobiotics as a substrate for the enzyme glutathione-S-transferase. The well documented tolerance of maize to the triazine herbicides is the result of conjugation of GSH to the herbicide. GSH is also the precursor of the phytochelatins that act as heavy metal binding peptides in plants.

The synthesis and degradation of GSH occurs continuously through the glutamyl cycle that has been well characterised in animals and at least portions have been confirmed in plants.



The first step in GSH synthesis (reaction 27) is the combination of glutamate and cysteine to form glutamylcysteine by the enzyme glutamylcysteine synthetase. The subsequent step involves the addition of glycine by the enzyme glutathione synthetase (reaction 28). In the legumes that accumulate hGSH, this second step involves the addition of alanine by the enzyme homogluthathione synthetase (reaction 29).

(27) Glu + Cys Glu-Cys

(28) Glu-Cys + Gly Glu-Cys-Gly

(29) Glu-Cys + Ala Glu-Cys-Ala

The degradation of GSH involves first the cleavage of the bond between glutamate and cysteine by glutamyl transpeptidase and the transfer of the glutamate residues to an acceptor amino acid:

Subsequently the Cys-Gly dipeptide is degraded by dipeptidases and Glu-aa by glutamylcyclotransferase: Animals contain a selenium containing enzyme, glutathione peroxidase, that reduces hydrogen peroxide forming GSSG and thereby serves as an alternative means of detoxifying activated oxygen. This enzyme was thought to be absent from higher plants but recently there have been reports of glutathione peroxidase in cultured cells. A plant cDNA showing homology to animal glutathione peroxidase has been isolated from *Nicotiana glauca*.

### **CONCLUSIONS ON OXIDATIVE STRESS:**

Oxygen free radicals or activated oxygen has been implicated in diverse environmental stresses in plants and animals and appears to be a common participation in most, if not all, degenerative conditions in eukaryotic cells. The peroxidation of lipids, the cross-linking and inactivation of proteins and mutations in DNA are typical consequences of free radicals, but because the reactions occur quickly and often are components of complex chain reactions, we usually can only detect their "footprints". Activated forms of oxygen are important in the biosynthesis of "complex" organic molecules, in the polymerisation of cell wall constituents, in the detoxification of xenobiotic chemicals and in the defence against pathogens. Thus, the plant's dilemma is not how to eliminate the activation of oxygen, but how to control and manage the potential reactions of activated oxygen. Complex systems of scavenging activated oxygen therefore exist in plant cells with complimentary and interdependent strategies. Some components such as the carotenoids prevent the formation of activated oxygen by competing for the energy leaked from the photosystems. Other components are lipid soluble and reside in the membrane bilayer to terminate the lipid peroxidation chain reactions. Still others, ascorbate and glutathione, are aqueous scavenger that detoxify activated oxygen directly or serve to recycle other protective components back to their reduced state. The enzymes that catalyse the synthesis, degradation and recycling of these antioxidants are essential to viability. Consequently they are highly conserved among plants, and exist in multiple forms in different subcellular compartments and different tissues to allow precise regulation.

## **ROLE OF OXIDATIVE STRESS IN DIABETES MELLITUS**

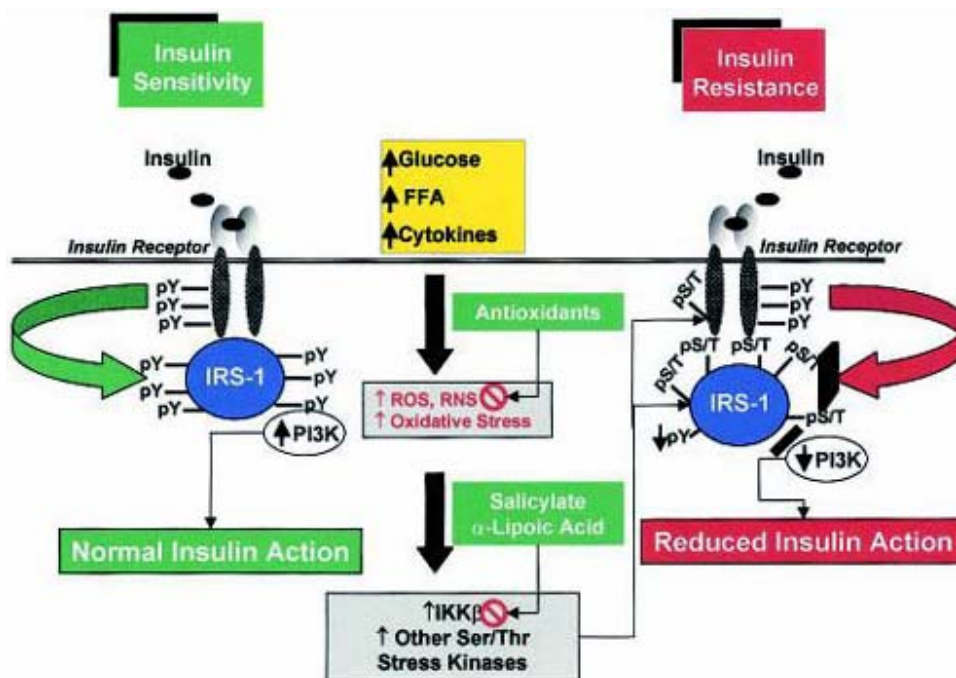
Diabetes mellitus is a multi-factorial disease in which increased oxidative stress plays an important pathogenic role. The term oxidative stress has been coined to represent a shift towards the pro-oxidant in the Pro-oxidant/Antioxidant balance that can occur as a result of an increase in oxidative metabolism. Chemical compounds capable of generating potential toxic oxygen species referred as pro-oxidants and compounds scavenging them are referred as antioxidants. Biochemical defects related to all diabetic complications may arise from overproduction of reactive oxygen species/nitrogen species. Free radicals arise from radiation, environmental chemicals, cigarette smoke, and various other environmental sources. A free radical is an atom or molecule that has one or more unpaired electrons, its tendency to acquire an electron from other anion for e.g.) hydroxyl radical, singlet oxygen, hydrogen peroxide, chlorine halogen radicals. When free radicals are overloaded, the antioxidants are not able to compete them and that leads to cell injury causing molecules to lose their structure and functions. ROS(reactive oxygen species) induced by elevation of glucose and free fatty acid levels, directly damage DNA, proteins, lipids, decrease insulin mRNA, cytosolic ATP, mitochondria, calcium influx into cytosol, and causes apoptosis. They also directly induce damage to tissues by activating number of stress sensitive signaling pathways (NFkB, P38 mitogen activated protein kinase, NH2 terminal junk kinase, protein kinase, stress activated protein kinase, hexosamines etc. Oxidative stress participates not only in beta cell dysfunction and insulin resistance but also in the genesis of late complications of diabetes. Oxidative stress /nitrosative stress also inducts formation of advanced glycation end products (AGE). Effect of advanced glycation end products on vascular structures is important in the pathogenesis of diabetic micro and macro vascular complications. Oxidative stress involves altering mitochondrial function, ion channel alteration, and abnormal growth factor signaling.

### **OXIDATIVE STRESS AND INSULIN RESISTANCE**

Both insulin resistance and decreased insulin secretion are major features of the pathophysiology of type 2 diabetes. Insulin resistance most often precedes the onset of type 2 diabetes by many years, is present in a large segment of the general population, and is multifactorial. It is clear that insulin resistance has a genetic component: insulin resistance is a feature of the offspring of parents with type 2 diabetes, aggregates in families, and, in longitudinal studies of families, has been implicated as a major risk factor for developing type 2 diabetes.

Insulin resistance is also caused by acquired factors, such as obesity, sedentary lifestyle, pregnancy, and the presence of excess hormones. Initially, insulin resistance is compensated for by hyperinsulinemia, through which normal glucose tolerance is preserved. Reaven <sup>[32]</sup> and others have reported that at least 25% of nondiabetic individuals exhibit insulin resistance that is in the range of that seen in patients with type 2 diabetes.

Deterioration into impaired glucose tolerance occurs when either the insulin resistance increases or the compensatory insulin secretory responses decrease, or when both occur. An increase in insulin, FFA, and/or glucose levels can increase ROS production and oxidative stress as well as activate stress-sensitive pathways. This, in turn, can worsen both insulin action and secretion, thereby accelerating the progression to overt type 2 diabetes.



**Figure 6.** The role of serine kinase activation in oxidative stress-induced insulin resistance. A variety of stimuli, including hyperglycemia, elevated FFA levels, cytokines, and others, increase ROS (and RNS) production and oxidative stress. This results in the activation of multiple stress-sensitive serine/threonine (Ser/Thr) kinase signaling cascades such as IKK- $\beta$  and others (see text for details). Once activated, these kinases are able to phosphorylate multiple targets, such as the IR and IRS proteins (including IRS-1 and IRS-2). Increased phosphorylation of IR or IRS proteins on discrete serine or threonine sites (pS/T) decreases the extent of insulin-stimulated tyrosine phosphorylation (pY). Consequently, the association and/or activities of downstream signaling molecules (e.g., phosphatidylinositol 3-kinase [PI3K]) are decreased, resulting in reduced insulin action (insulin resistance). The protective effects of antioxidants (e.g., LA) on oxidative stress-induced insulin resistance could relate to their ability to preserve the intracellular redox balance (neutralizing ROS) or, analogous to pharmacological agents (e.g., salicylates, p38 MAPK inhibitors), to block the activation of stress-sensitive kinases.

The role of serine kinase activation in oxidative stress-induced insulin resistance. A variety of stimuli, including hyperglycemia, elevated FFA levels, cytokines, and others, increase ROS (and RNS) production and oxidative stress. This results in the activation of multiple stress-sensitive serine/threonine (Ser/Thr) kinase signaling cascades such as IKK- $\beta$  and others (see text for details). Once activated, these kinases are able to phosphorylate multiple targets, such as the IR and IRS proteins (including IRS-1 and IRS-2). Increased phosphorylation of IR or IRS proteins on discrete serine or threonine sites (pS/T) decreases the extent of insulin-stimulated tyrosine phosphorylation (pY)[52,53]. Consequently, the association and/or activities of downstream signaling molecules (e.g., phosphatidylinositol 3-kinase [PI3K]) are decreased, resulting in reduced insulin action (insulin resistance). The protective effects of antioxidants (e.g., LA) on oxidative stress-induced insulin resistance could relate to their ability to preserve the intracellular redox balance (neutralizing ROS) or, analogous to pharmacological agents (e.g., salicylates, p38 MAPK inhibitors), to block the activation of stress-sensitive kinases.

## **MECHANISMS FOR INCREASED OXIDATIVE STRESS IN DIABETES**

### **ADVANCED GLYCATION ENDPRODUCTS**

Advanced glycation or glycosylation endproducts (AGEs) are the products of glycation and oxidation (glycoxidation), which are increased with age, and at an accelerated rate in diabetes mellitus.

In vitro studies have suggested that glycation itself may result in production of superoxide. Oxidation has been hypothesized to result in generation of superoxide, H<sub>2</sub>O<sub>2</sub> and through transition metal catalysis, hydroxyl radicals. Catalase and other antioxidants decrease cross linking and AGE formation.

### **Alterations in glutathione metabolism**

Tissue glutathione plays a central role in antioxidant defense. Reduced glutathione detoxifies reactive oxygen species such as hydrogen peroxide and lipid peroxides directly or in a glutathione peroxidase (GPX) catalyzed mechanism. Glutathione also regenerates the major aqueous and lipid phase antioxidants, ascorbate and  $\alpha$ -tocopherol. Glutathione reductase (GRD) catalyzes the NADPH dependent reduction of oxidized glutathione, serving to maintain intracellular glutathione stores and a favorable redox status. Glutathione-S-transferase (GST) catalyzes the reaction between the -SH group and potential alkylating agents, rendering them more water soluble and suitable for transport out of the cell. GST can also use peroxides as a substrate.

### **Glutathione homeostasis**

Type 2 diabetic patients had decreased erythrocyte GSH and increased GSSG levels.

Blood GSH was significantly decreased in different phases of type 2 DM such as: glucose intolerance and early hyperglycemia, within two years of diagnosis and before development of complications and in poor glycemic control. Red cells from type 2 DM patients had decreased GSH levels, impaired gamma-glutamyl transferase activity and impaired thiol transport. Treatment with an antidiabetic agent for 6 months corrected these changes.

An inverse correlation between erythrocyte GSH levels and the presence of DM complications in type 1 and 2 DM patients. However, most studies have also found decreased blood or red cell glutathione levels in type 2 DM patients. Less firm conclusions can be drawn in type 1 DM patients. It has to be clarified whether the levels are decreased in patients without complications and whether patients with complications have even lower levels. The pathophysiological significance of decreased glutathione levels in DM remains to be shown.

### **Glutathione dependent enzymes**

No difference in whole blood GRD activity in type 1 and type 2 DM patients compared to non-diabetic control patients. Also found normal red cell GRD enzyme kinetics in type 1 DM patients. On the other hand, blood GRD activity was lower in children with type 1 DM compared to healthy children.

A large number of studies have shown that red blood cell, whole blood and leukocyte, glutathione peroxidase (GPX) activity was similar in type 1 and type 2 DM patients compared to control groups. On the other hand, erythrocyte GPX activity was also impaired in Asian diabetic patients. In type 1 DM plasma selenium levels were normal, but red cell selenium content and GPX activity were decreased.

Normal red cell GST enzyme kinetics were found in type 1 DM patients. GST activity has been reported to be decreased in heart and liver. Changes in glutathione dependent enzymes in experimental diabetic models have been contradictory. Most studies show tissue and time dependent changes in enzyme activity. Even taking these factors into account, no consensus can be found among studies about the impact of DM on glutathione dependent enzyme activity. Changes in glutathione dependent enzymes in diabetic patients are also inconsistent. Differences in results cannot be completely explained by study methodology.

### **Impairment of superoxide dismutase and catalase activity**

Superoxide dismutase (SOD) and catalase are also major antioxidant enzymes. SOD exists in three different isoforms. Cu,Zn-SOD is mostly in the cytosol and dismutates superoxide to hydrogen peroxide. Extracellular (EC) SOD is found in the plasma and extracellular space. Mn-SOD is located in mitochondria. Catalase is a hydrogen peroxide decomposing enzyme mainly localized to peroxisomes or microperoxisomes. Superoxide may react with other reactive oxygen species such as nitric oxide to form highly toxic species such as peroxynitrite, in addition to direct toxic effects.

Peroxynitrite reacts with the tyrosine residues in proteins resulting with the nitrotyrosine production in plasma proteins, which is considered as an indirect evidence of peroxynitrite production and increased oxidative stress. Although nitrotyrosine was not detectable in the plasma of healthy controls, nitrotyrosine was found in the plasma of all type 2 diabetic patients examined. Consistent with these results, plasma nitrotyrosine values were correlated with plasma glucose concentrations. Furthermore, exposure of endothelial cells to high glucose leads to augmented production of superoxide anion, which may quench nitric oxide. Decreased nitric oxide levels result with impaired endothelial functions, vasodilation and delayed cell replication. Alternatively, superoxide can be dismutated to much more reactive hydrogen peroxide, which through the Fenton reaction can then lead to highly toxic hydroxyl radical formation. Decreased activity of cytoplasmic Cu,Zn-SOD and especially mitochondrial (Mn-) SOD in diabetic neutrophils was found. Consequently superoxide levels as estimated indirectly by cytochrome c reduction were elevated in neutrophils from diabetic patients as a result of decreased SOD activity. Major reason for the decreased SOD activity is the glycosylation of Cu,Zn-SOD which has been shown to lead to enzyme inactivation both in vivo and in vitro. Also Cu,Zn-SOD cleavage and release of Cu<sup>++</sup> in vitro resulted in transition metal catalyzed ROS formation.

Erythrocyte Cu,Zn-SOD activity correlated inversely with indices of glycemic control in DM patients, however. Red cell Cu,Zn/SOD activity has also been found to be decreased in DM patients. Glycation may decrease cell-associated EC-SOD, which could predispose to oxidative damage. Jennings et al. Decreased red cell Cu,Zn-SOD activity in type 1 DM patients with retinopathy compared to type 1 DM patients without microvascular complications and non-diabetic control subjects. However, there are reports disagreeing with these findings. Red cell Cu,Zn-SOD activity was similar in type 1 and 2 DM patients compared to normal subjects, irrespective of microvascular complications. Leukocyte SOD activity was similar between type 2 DM patients and healthy control subjects, despite increased lipid peroxidation and decreased ascorbate levels. Furthermore, increased red cell SOD activity and serum MDA levels were reported in patients of type 1 DM with normo- microalbuminuria and retinopathy compared to healthy subjects.

Red cell superoxide and catalase activities were decreased in 105 subjects with impaired glucose tolerance (IGT) and early hyperglycemia and also in type 2 DM patients. However, in another study red cell catalase and SOD activities were normal in 26 type 2 DM patients in poor glycemic control. EC-SOD activity was found to be similar in type 1 DM patients, despite somewhat higher plasma EC-SOD levels.

The wide variability among studies does not allow conclusions to be drawn as to whether SOD isoform or catalase enzyme activities are abnormal in diabetic patients. Again, differences in methodology or study design do not completely explain the conflicting findings among studies.

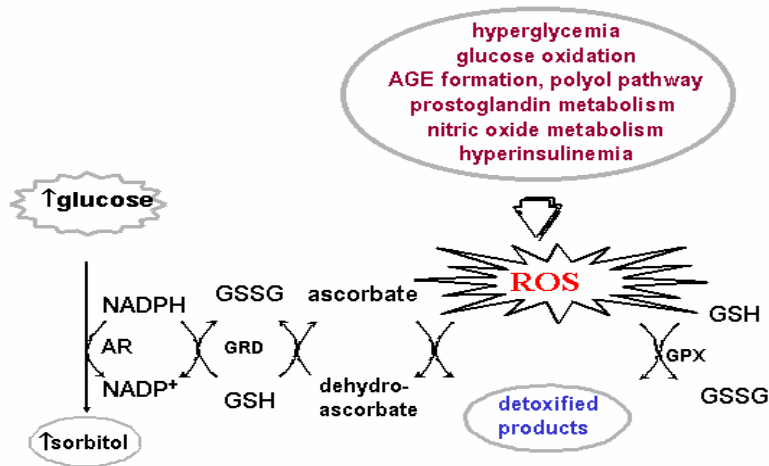


Figure 7.

### The polyol pathway

Hyperglycemia induces the polyol pathway, resulting in induction of aldose reductase and production of sorbitol. Importance of the polyol pathway may vary among tissues. Induction of oxidative stress may occur through many different mechanisms, including depletion of NADPH and consequent disturbance of glutathione and nitric oxide metabolism. Mean red cell GSH and NADPH levels and NADPH/NADP<sup>+</sup> and GSH/GSSG ratios were decreased in type 2 diabetic compared to non-diabetic.

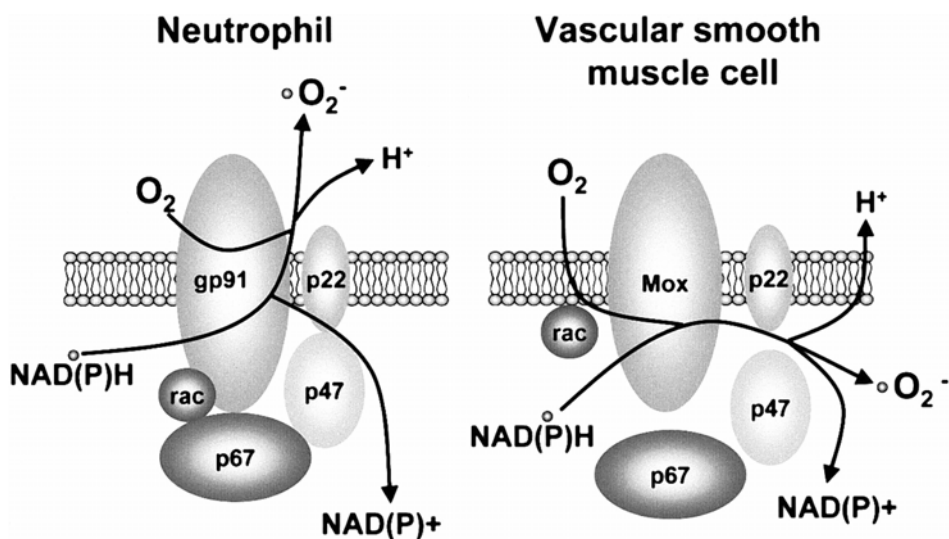


Figure 8.

One week of treatment with the aldose reductase inhibitor Tolrestat improved the NADPH and GSH levels in diabetes whose NADPH levels were depressed. Thus in at least a subset of type 2 DM activation of the polyol pathway appears to deplete erythrocyte NADPH and GSH. Similarly in a recent study aldose reductase inhibitor sorbinil restored nerve concentrations of antioxidants reduced glutathione (GSH) and ascorbate, and normalized diabetes-induced lipid peroxidation in streptozotocin-diabetic rats.

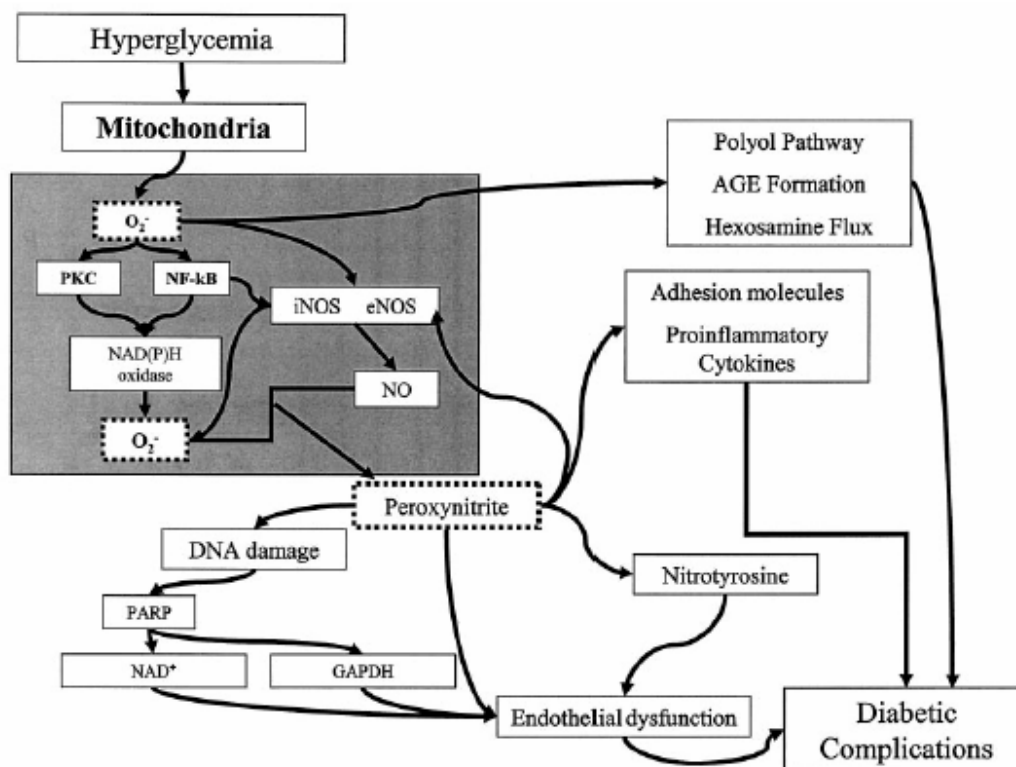
### **HYPERGLYCEMIA, HYPERPHOSPHATEMIA AND OXIDATIVE STRESS**

Hyperglycemia can increase oxidative stress through several pathways. A major mechanism appears to be the hyperglycemia-induced intracellular reactive oxygen species (ROS) and resulting in an increased production of superoxide. Chronic hyperglycemia and advanced glycation end products increase oxidative stress in the endothelial cells, resulting in lower NO availability, DNA damage and lipid oxidation, and eventually cause endothelial dysfunction. Oxidative stress is widely invoked as a pathogenic mechanism for atherosclerosis. Among the sequelae of hyperglycemia, oxidative stress has been suggested as a potential mechanism for accelerated atherosclerosis. Therefore, strategies to reduce postprandial hyperglycemia and hyperinsulinemia represent an important approach to improving glycemic control in patients with type 2 diabetes mellitus and may even prevent the deterioration of glucose metabolism in impaired glucose tolerance and the subsequent progression to diabetes. Both metabolic and epidemiologic evidence suggest that replacing high-glycemic-index forms of carbohydrate with low-glycemic-index carbohydrates could reduce the risk of type 2 diabetes.

### **POSTPRANDIAL HYPERGLYCEMIA AND OXIDATIVE/NITROSATIVE STRESS**

Recent studies demonstrate that hyperglycemia induces an overproduction of superoxide by the mitochondrial electron-transport chain. Superoxide overproduction is accompanied by increased NO generation, due to endothelial NO synthase (eNOS) and inducible NO synthase (iNOS) uncoupled state, a phenomenon favoring 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 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 (glyceraldehyde-3-phosphate dehydrogenase). These processes result in acute endothelial dysfunction in diabetic blood vessels that, convincingly, contributes to the development of CVD. These pathways are summarized in Fig. 9.





**Figure 9.**

In endothelial cells, glucose can pass freely, in an insulin-independent manner, through the cell membrane. Intracellular hyperglycemia induces overproduction of superoxide at the mitochondrial level. Overproduction of superoxide is the first and key event in the activation of all other pathways involved in the pathogenesis of diabetes complications, such as polyol pathway flux, increased advanced glycation end product (AGE) formation, activation of protein kinase C (PKC) and nuclear factor- $\kappa$ B (NF- $\kappa$ B), and increased hexosamine pathway flux.  $O_2^-$  reacting with NO produces peroxynitrite (ONOO<sup>-</sup>). Superoxide overproduction reduces eNOS activity but, through nuclear factor- $\kappa$ B and protein kinase C, activates NAD(P)H and increases iNOS expression; the final effect is an increased NO generation. This condition favors the formation of the strong oxidant peroxynitrite, which in turn produces, in iNOS and eNOS, an uncoupled state, resulting in the production of superoxide rather than NO, and 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 depletes the intracellular concentration of its substrate NAD<sup>+</sup>, slowing the rate of glycolysis, electron transport, and ATP formation and produces an ADP ribosylation of the GAPDH. This process results in acute endothelial dysfunction in diabetic blood vessels that contributes to the development of diabetes complications.

Nuclear factor- $\kappa$ B activation also induces a proinflammatory condition and adhesion molecules overexpression. All of these alterations produce the final picture of diabetes complications.

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