CHAPTER II

LITERATURE REVIEW

2.1 Overview of antioxidant activity, polyphenols in plants and product traditionally used in diabetes

2.1.1 Research on antioxidant activity, polyphenols in plants and product traditionally used in diabetes

There has been considerable interest in finding natural antioxidants from plant materials to replace synthetic ones. Natural antioxidant substances are accepted as safe since they originate from in plant foods. Information from both scientific reports and laboratory studies demonstrate that fruits and vegetables are a source of phytochemicals, or plant chemicals, such as carotene, anthocyanins and other polyphenolic compounds which are rich in antioxidant activity. Many studies have indicated that polyphenolic compounds (an example of the typical structure is shown in Table 2.1) reduce oxidative stress associated with chronic diseases such as diabetes [14, 28-33, 35-39]. Epidemiological studies have shown a correlation between an increased consumption of phenolic antioxidants and reduced risk of cardiovascular diseases [40]. The cardioprotective effects are increasingly suggested to stem

from the antioxidant capacity of the phenolic compounds [41]. They may contribute to chemoprevention of a variety of human diseases such as diabetes, cancer and also may be used for health promotion. Thus, there is potential benefit from the use of dietary biofactors in medicinal and food plants.

Flavonoids and other polyphenols belong to the recently popular phytochemicals, chemicals derived from plant material with potentially beneficial effects in human health. Flavonoids are an especially well-studied class of secondary metabolites. Many of them (Table 2.2), particularly the blue and red anthocyanidins and the yellow chalcones, flavones, and flavonols are conspicuous components of flowers, fruits, and other parts of plants. While most flavonoids exist as glucosides, aglycones (also important for health) are found in nature. Flavonoids are widely distributed in plants and also occur in mosses, rarely in fungi and lichens. Aside from flavonoids, the related class of tannins will be scrutinized, together with sample polyphenols, such as phenolic acids and synthetic compounds [10]. Their activities were reported to be against oxidative damage with aging or age-related neurodegenerative diseases. Studies reporting the mechanism of action of flavonoids in preventing ROS-induced oxidative stress have identified three different mechanisms: flavonoids can prevent cell death after glutamate injury by scavenging ROS, maintaining the correct GSH levels and inhibiting Ca^{2+} influx, which represents the last step in cell death cascade. These properties, coupled with the anti-inflammatory properties attributed to some phenolics, render this class of compounds suitable

for application against oxidative stress, as well as against inflammation and antioxidant defense depletion.

Table 2.1 Representations of the subclasses of polyphenolic compounds in plants [10]

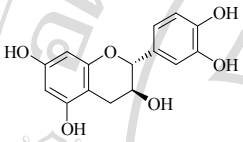
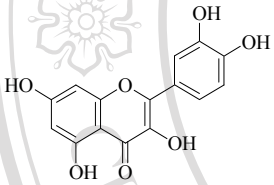
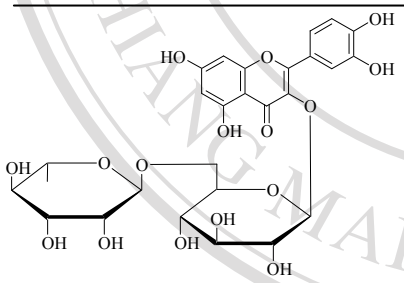
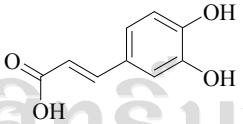
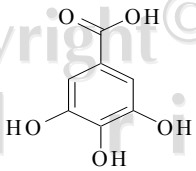
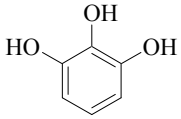
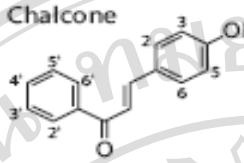
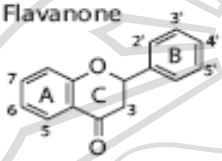
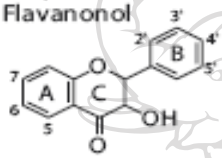
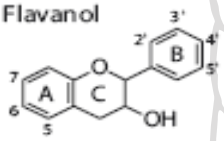
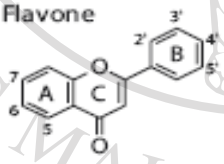
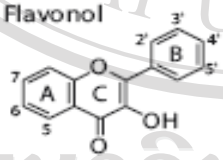
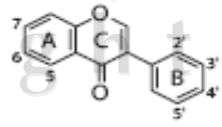
Structure formula	Class	Examples
	Flavonoids	Catechin
	Flavonoids	Quercetin
	Flavonoids	Rutin
	Hydroxycinnamic acids	Caffeic acid
	Phenols	Gallic acid
	Phenols	Pyrogallol

Table 2.2 The different subclasses of flavonoids [10]

Structure formula	Representations
Chalcone 	Butein Okanin
Flavanone 	Eriodictyol Naringenin Naringin Hesperitin Prunin
Flavanonol 	Taxifolin Pinobanksin
Flavanol 	Catechin Epicatechin EGC ECG
Flavone 	Apigenin Luteolin Chrysin Tricetin Isovitexin
Flavonol 	Quercetin Kaempferol Myricetin Rhamnetin Myricitrin Quercitrin Morin Isorhamnetin
Isoflavone 	Daidzein Daidzin Genistein Genistin
Anthcyanidin 	Cyanidin Pelargonidin Delphinidin Petunidin Malvidin

Catechins and proanthocyanidins also exhibit powerful antioxidant activities [42-43]. Catechin and its derivative, epigallocatechin from green tea, are known to attenuate ROS-mediated cerebral ischemia/reperfusion injuries in animals and the oligomeric proanthocyanidins can strengthen collagen. This property together with antioxidant capabilities help protect against degeneration of connective tissues, bones, gums, eyes, skin [44], prevent premature aging, relieve premenstrual syndrome [45] and also varicose vein pain and swelling. Catechin has also been found to increase the activity of respiratory enzymes of liver cells [46]. Both catechins and proanthocyanidins possess the ability to protect connective tissue against oxidative damage, the linings of the intestine and the stomach and even the vertebral spaces [47]. Proanthocyanidins (oligomeric) have also been reported to provide protection against edema [48] and against inflammatory conditions and allergic reactions. Grape seed proanthocyanidin extracts which contain a combination of biologically active flavonoids have been demonstrated to improve the cardiac functional assessment including post ischemic left ventricular function, reduced myocardial infarct size and tachycardia [42]. Besides, Yamakoshi et al. [49] indicated that procyanidin-rich grape seed extract may contribute significantly to the prevention of cataracts by their antioxidant action. Chronic inflammation is widely argued to be an underlying biological mechanism responsible for physical function decline in the elderly. Age-related diseases such as AD, PD, atherosclerosis, type 2 diabetes, sarcopenia, osteoporosis, cognitive decline and

frailty are initiated or worsened by systemic inflammation and therefore shorten the survival rate of humans [43-50] . Epidemiological studies have shown a correlation between an increased consumption of phenolic antioxidants and reduced risk of cardiovascular diseases [40]. The cardioprotective effects are increasingly suggested to stem from the antioxidative capacity of the phenolics [41]. Potentials of epicatechin gallate, epigallocatechin gallate and epigallocatechin can reduce atherosclerotic lesions in various animal models mainly by protecting LDL oxidation against oxidative stimuli [51-52]. Extracts of *Crataegus monogyna* have long been used for the treatment of mild heart disease. The polyphenolic content and antioxidant potentials of *C. monogyna* characterized by other clinical studies have demonstrated that fatty streak formation in aortas of hamsters is greatly reduced when treated with catechin hydrate along with vitamin E [53]. Glabridin, an isoflavan, reduces the susceptibility of LDL to oxidation thus showing an inhibitory effect on atherogenesis [54]. Red wine polyphenolic compounds have been shown to have antioxidant properties protecting against coronary heart disease, and confer protect[tion by sparing the endogenous LDL α-tocopherol as well as preserving normal vascular activity by acting at different stages of the cascade that leads to lipid peroxidation, endothelium dysfunction and vasospasm [42, 55-59].

Similarly, several experimental studies have been carried out to demonstrate the beneficial effects of polyphenols on diabetes. Phytoestrogens

such as soya isoflavones genistein and daidzein have been found to exert some effects on the sodium-dependent glucose transporter of the intestine, inhibiting glucose uptake, thus, reducing blood glucose levels. The isoflavones can even act as potent antioxidants to protect against glucose-induced LDL oxidation [60]. Both effects are therefore likely to confer protection against diabetes and its associated complications. Hermansen et al. [61] indicated the beneficial effects of soy-based dietary supplement on lipid level and cardiovascular risk markers in type 2 diabetic subjects. The effects of the isoflavones and oligomeric proanthocyanidins compounds in reducing diabetic retinopathy are of considerable interest. Vinson and Zhang [62] reported that polyphenolic rich black and green teas significantly inhibited diabetic cataracts in the streptozotocin-induced rat model of diabetes. This beneficial effect of tea can be ascribed to the presence of the bioactive catechin derivative, epigallocatechin gallate which has been shown to modify glucose and lipid metabolism in H411E rat hepatoma cells while enhancing glucose tolerance in diabetic rodents [63]. Chlorogenic acid has also been reported to improve glucose tolerance in vivo while derivatives of chlorogenic acid have been reported as hypoglycemic agents and are of interest due to their association with diabetes mellitus [64]. In addition, ferulic acid has shown antihyperglycemic effects as well as it increased the activities of SOD, catalase, glutathione peroxidase thereby enhancing the endogenous antioxidant capacity of diabetic animals [65]. The anthocyanins, cyanidin-3-glucoside and

delphinidin-3-glucoside have been found to be effective insulin secretagogues in rodent pancreatic β-cells in vitro. This suggests that anthocyanin-rich fruits and vegetables and other dietary phenolic compounds may have clinical application in the management of diabetes [66].

Many previous ethnopharmacological studies showed that therapeutic agents from plants had the potential to reduce the oxidative damage in diabetic animals [26, 67-71]. Quercetin, a constituent present in fruits and vegetables, ameliorated the diabetes-induced changes in oxidative stress. When the compound was used at two different doses for 45 days, it reduced the levels of blood glucose, plasma TBARS and hydroperoxides. It also resulted in the activities of SOD and CAT decreasing to near normal level. Quercetin at lower doses was found to be more effective [72].

In a recent study, the edible fruit tissue of the *Phyllanthus emblica* Linn. (PE), a member of the Euphorbiaceae, contained about three times as much protein and 160 times as much ascorbic acid as apples (*Malus pumila* Mill.). The fruits also contained considerably higher concentrations of most minerals and amino acids than apples. Glutamic acid, proline, aspartic acid, alanine and lysine constituted 29.6, 14.6, 8.1, 5.4 and 5.3%, respectively, of the total amino acids. The concentration of each amino acid, except cystine, was much higher than in apples. The PE is highly nutritious, and could be an important dietary source of vitamin C, minerals and amino acids [73]. PE contains a lot of tannins and flavonoids which possess antioxidant activity [74]. A high

correlation coefficient exists between the phenolic content and superoxide anion radical scavenging activity [75]. Rao et al. studied the efficacy of PE for relieving the oxidative stress and improving glucose metabolism in streptozotocin-induced diabetic rats. PE extracts 20 or 40 mg/kg of body weight/day) or a polyphenol-rich fraction of ethyl acetate extract (10 or 20 mg/kg of body weight/day) was given orally for 20 days to the rats. PE extracts showed strong free radical scavenging activity by reduced lipid peroxidation (TBARS) and strong inhibition of the production of advanced glycosylated end products by reduced 5-hydroxymethylfurfural, dose-dependently and significantly. Also PE decreased serum blood glucose in diabetic rats [76].

Chaiyasut & Chansakaow (2007) studied the inhibitory effects of *Phyllanthus emblica* Linn. and *Kaempferia parviflora* (KP) extracts. The plant extracts were investigated for their inhibitory activities on AAPH-induced protein oxidation to allophycocyanin. The loss of allophycocyanin fluorescence was measured and interpreted as a half-life of the protein. The results of 1.0 µg/ml of PE and KP were 21.24 and 11.31 minutes. The assay of protein glycation at 37 ^{o}C for 2 weeks was also performed. Results reported PE achieved 88.09 % inhibition against protein glycation formation at 10.0 µg/ml whereas, KP achieved 28.10 % inhibition at 50 µg/ml. Thus, the extracts had potential anti-aging use from oxidative protein damage [77].

The effects of *Terminalia chebula* fruit extract (TC) on the levels of plasma and tissue glycoprotein components in streptozotocin-induced-diabetic

rats was studied [78]. It was found that oral administration of TC at a concentration of 200 mg/kg body weight for 30 days significantly reduced the levels of blood glucose, glycosylated hemoglobin, urea, and creatinine as well as sucrose, hexose, hexosamine and sialic acid in the diabetic rats. The levels of plasma insulin and C-peptide in the diabetic rats were elevated to near normal by TC treatment. By the histological observations, it was found that pancreatic tissue of control and experimental groups also revealed the beneficial effects of TC. The TC fruit extract had comparable efficacy compared to glibenclamide.

Polyphenolic compounds in crude extracts of root, leaf and fruit of *Morinda citrifolia* (MC) were analyzed. The results exhibited considerably high antioxidative activity by ferric thiocyanate assay and thiobarbituric acid test. The activities of some MC fractions were sensitive to those of either tocopherol or BHT. However, the different parts of plants were found to contain different amounts of total phenolic compounds which involve various mechanisms inhibiting the oxidation process and contributed significantly to antioxidation, which is important in combating oxidative stress [79].

The antioxidative activities of the medicinal plant, *Houttuynia cordata* and its compounds was investigated by using the thiocyanate method to evaluate inhibitory effects on lipid peroxidation in the linoleic acid system [80]. The peroxide levels slowly increased during incubation in the presence of linoleic acid over 3 days, and the plant reduced lipid peroxidation more effectively as lipid peroxidation progressed, resulting in inhibition of about

80% relative to the control value by the 3rd day of incubation. In addition, polyphenols isolated from the plant also showed marked and dose-dependent inhibitory effects on lipid peroxidation. The compounds with the strongest activities were 3, 4-dihydroxylbenzoic acid, quercetin, the quercetin glycosides quercetin-3-O-β-D-galactoside, quercetin-3-O-α-L-rhamnoside, quercetin-3-O-β-D-glucoside and quercetin-3-O-rutinose, catechin, gallic acid, methyl gallate and rosamultin isolated from *Houttuynia cordata.* Moreover, quercetin glycosides showed stronger activity than quercetin, suggesting that its glycosylation increases the antioxidative activity of quercetin. The results indicate that the medicinal plant and their polyphenols show their potential therapeutic use for various disorders involving free radical reactions [80].

A review of traditional medicinal plants with anti-diabetic potentials done in India [81] showed that administration of Ivy Gourd (*Coccinia grandis* Voigt. Syn.) extract (200 mg/kg body weight) for 45 days reduced blood sugar in diabetic rats and have been potent antioxidant activity [82-84]. In addition, , the aqueous fruit extract of *Aegle marmelos* Coorea (250 mg/kg, twice daily for one month) produces an anti-hyperglycemic effect along with decreasing glycosylated haemoglobin level in STZ induced diabetic albino wistar rats [85]. Hypoglycemic and antioxidant activity of the leaves had been observed in diabetic male albino rats [86] and antioxidant activity in STZ diabetic rats along with partial repair of damaged pancreatic islets [87]. Treatment of severely diabetic rats (fasting blood glucose level >250 mg/ml) for 14 days

with aqueous extract (250mg/kg, orally) of *Aegle marmelos* seeds reduced the fasting blood glucose by 60.84% and urine sugar by 75% than their pretreatment levels [88].

Miura et al. reported that aqueous extract of *Momordica charantia* Linn. fruit (200 mg/kg, orally for 6 weeks) and exercise, potentially lowered blood sugar of type 2 diabetic and hyperinsulinemic (insulin resistance) rats [89]. Aqueous extract of their seeds produced prominent reduction in blood glucose, glycosylated hemoglobin, lactate dehydrogenase, glucose-6-phosphatase, fructose-1, 6 bisphosphatase and glycogen phosphorylase along with increased hemoglobin, glycogen content. The results implied that it had the potential effect to be a part of a dietary supplement for diabetic patients [90].

Chandra et al. showed the oral treatment of diabetic rats with *Allium sativum*, *Momordica charantia* and *Ocimum sanctum* extracts (500 mg/kg of body weight) not only decreased the blood glucose level but also inhibited the formation of lipid peroxides that led to decrease oxidative load in diabetes, reactivated the antioxidant enzymes, and enhanced levels of GSH and metals. The herbal extracts (50–500 μg) inhibited the generation of superoxide anions ($O_2^{-\cdot}$) in both enzymatic and nonenzymatic *in vitro* systems. These preparations also inhibited the ferrous-sodium ascorbate-induced formation of lipid peroxides in red blood cells. The results are not only useful in controlling oxidative stress but are also helpful in further strengthening the antioxidant potential. [11,91-92]. In addition, Jelodar et al. found beneficial effects of

Allium sativum on blood glucose and histopathology of the pancreas of alloxan-induced diabetic rats after feeding a diet containing *Allium sativum* Linn. (12.5%) for 15 days. The results showed a reduction in blood glucose as compared to the control group [93]. Furthermore, oral administration of a laboratory diet containing 0.05% of ajoene (derived from *Allium sativum*) for 8 weeks has been observed to produce an anti-diabetic effect in genetically diabetic KK-A(y) mice. The levels of plasma glucose was significantly by suppressed 73.8% [94]. S-allyl cysteine, a key component of aged *Allium sativum*, is a potent antioxidant and inhibited AGEP (accumulation of advanced glycation end products) formation [95].

Numerous plant products have been used as natural folk medicines worldwide. Scientists have increased interest in medicinal plants as they recognize the health benefits of these products. While searching for food, it was found that some foods had specific properties of relieving or eliminating certain diseases and maintaining good health. It was the beginning of the use of herbal medicine [96]. Many studies have demonstrated benefits of plant products, such as a study in China showing that an herbal formula, Jin-Qi-Jiang-Tang-Pian (JQJTP) was able to decrease blood glucose, triglyceride and malondialdehyde (MDA) significantly in alloxan-induced diabetic rats for two months; with an increase of the superoxide dismutase (SOD)/MDA ratio, as compared with control groups [97]. In addition, Hachimi-jiogan, a cocktail comprising 8 kinds of medicinal plants, was orally administered to the

streptozotocin (STZ)-induced diabetic rats for 10 days, counteracting oxidative stress in a dose-dependent manner. The levels of blood glucose and glycosylated compounds were also decreased in the treated group. Dose-dependent reduction of elevated urinary protein content, superoxide and nitrite/nitrate was observed. Moreover, thiobarbituric acid-reactive substance (TBARS) levels in serum, hepatic and renal mitochondria were dose-dependently reduced in the treated groups compared to the control diabetic group [98].

2.1.2 Beneficial and safety of BFPB with probiotics

Biologically fermented plant beverage (BFPB), a probiotic liquid product, is slightly sour and clear brown. It is made from different kinds of plants, herbs, vegetables or fruits by fermentation with lactic acid-producing or probiotic bacteria under an anaerobic environment. These fermented foods have been used for food preservation, enhancing health and improving nutrition. BFPB has strong antioxidant activity compared to vitamin C and Trolox and effectively to scavenges free radicals, inhibiting lipid peroxidation and protects against oxidative DNA damage [99-100]. The benefit of BFPB is derived from the synergy of plant chemicals with bioactive compounds and probiotics [101-102]. Probiotics are live microorganisms, including *Lactobacillus* species, which have health benefits to the host when

administered in adequate amounts. Their products are widely used in various forms because of their multiple biological effects and have potential applications to various diseases. They have been widely and have undergone the several clinical tests [103]. Previously, in Japan, Minamiyama et al. evaluated the effect of antioxidant biofactor (AOB), a fermented-grain food supplement, a mixture of commercially available fermented grain foods that have strong antioxidant activity, on oxidative stress in type 2 diabetic rats. The results showed that the AOB product decreased blood glucose, hemoglobin A1c, triacylgycerol, low density lipoprotein, cholesterol and PAI-1 and AOB significantly improved the NO-cGMP pathway via normalizing ROS generation in diabetic rats. The data suggest that dietary supplementation with AOB contributes to nutritional strategies for the prevention and treatment of type 2 diabetes [104]. Furthermore, Datla et al. studied the use of antioxidants as protective agents in disease associated with increased oxidative stress. The effect of a natural antioxidant drink, EM-X (a ferment derivative of unpolished rice, papaya and seaweed with effective microorganisms), was investigated by using the rat model of Parkinson's disease. The evidence presented supports the potential protective effects of EM-X drink on oxidative stress [105]. In addition, EM-X containing lactic acid bacteria, yeast and photosynthetic bacteria (containing minerals, α-tocopherol, lycopene, ubiquinone, saponin and flavonoids) was found to have a beneficial effect on the aging process associated with bone loss of the femur in rats [106].

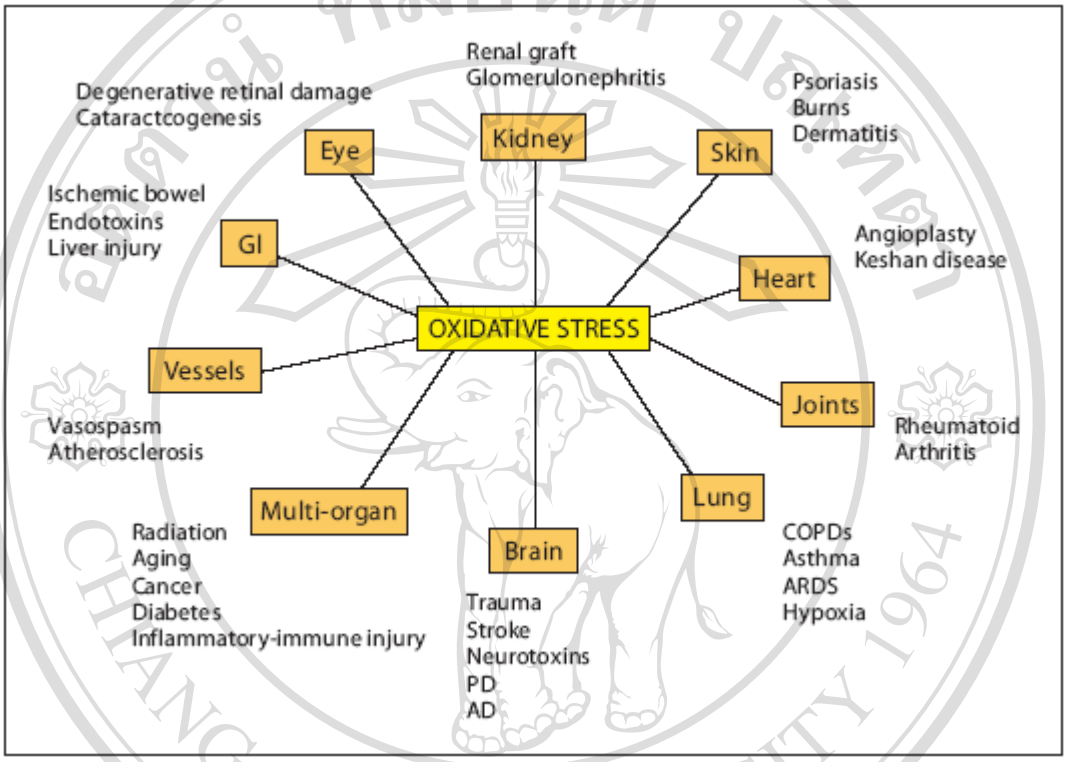
Safety and effects of BFPB or lactic acid beverages fermented with *Lactobacillus* spp. and organic material is now available in Thailand. It is believed to relieve disease symptoms and improve overall health. Previous studies reported that BFPB demonstrated hydrophilic antioxidant capacity and free phenolic antioxidant capacity, representing the major contribution for antioxidative property [107], antibacterial activity, and anti-inflammation [102, 108]. However, Biologically fermented products were established and have been studied until now. Research on the safety of consumption were previously started. The studies of constituents, chemical and biological properties of biologically fermented products showed a manufacture related with the safety of product [108-109]. Pasteurization was added to their manufacturing. Moreover, those processes which gave the safety product were produced the biologically fermented products to animal experimentation. Sub-chronic toxicity examined on sprague dawley rats which were administered daily biologically fermented products for 60 days at dose of 1.2 and 9.0 ml/kg BW/day, equivalent to 1 and 7.5 fold of effective dose, repectively, including the post-effective dose that the animals were orally dose at 1.2 ml/kg BW/day but they were stopped the experiment for 7 days before sacrifice. *Morinda citrifolia* Linn., *Houttuynia cordata* Thunb., and *Phyllanthus emblica* Linn. which fermented with *Lactobacillus plantarum* for 30 days were treated-substances. All of the rats had no detectable behavior or activity change, and no diarrhea or death occurred. It was found that almost all male rats treated with

biologically fermented products had more body weight gain than all female rats. While statistical analysis showed some significant difference, the studies, including heamatological studies, blood chemistry studies and histopathological examination, have shown no toxicity to the animals [109]. In addition, the study of fermentation kinetics of *Morinda citrifolia* Linn and antimicrobial activity of its products results revealed the safety of fermented products for consumption [110]. In addition, an acute toxicity test has shown that this product could be safely ingested by mice and that live cells labeled with a fluorescent dye persist in the mouse gastrointestinal tract for at least 7 days after orogastric intubation [108].

2.2 Oxidative Stress

Oxidative stress is caused by an imbalance between the production of reactive oxygen (or nitrogen) species (ROS/RNS) 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, diabetes and the process of aging, which are associated with chronic inflammatory reactions (Scheme 2.1).



Scheme 2.1 Schematic representation of the involvement of oxidative stress in several clinical conditions most of which have underlying inflammatory mechanisms. ARDS = Adult respiratory distress syndrome; COPD = chronic obstructive pulmonary disease. AD = Alzheimer's disease; PD = Parkinson's disease [10].

Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron (free radicals may also be formed by the collision of the non-radical species by a reaction between a

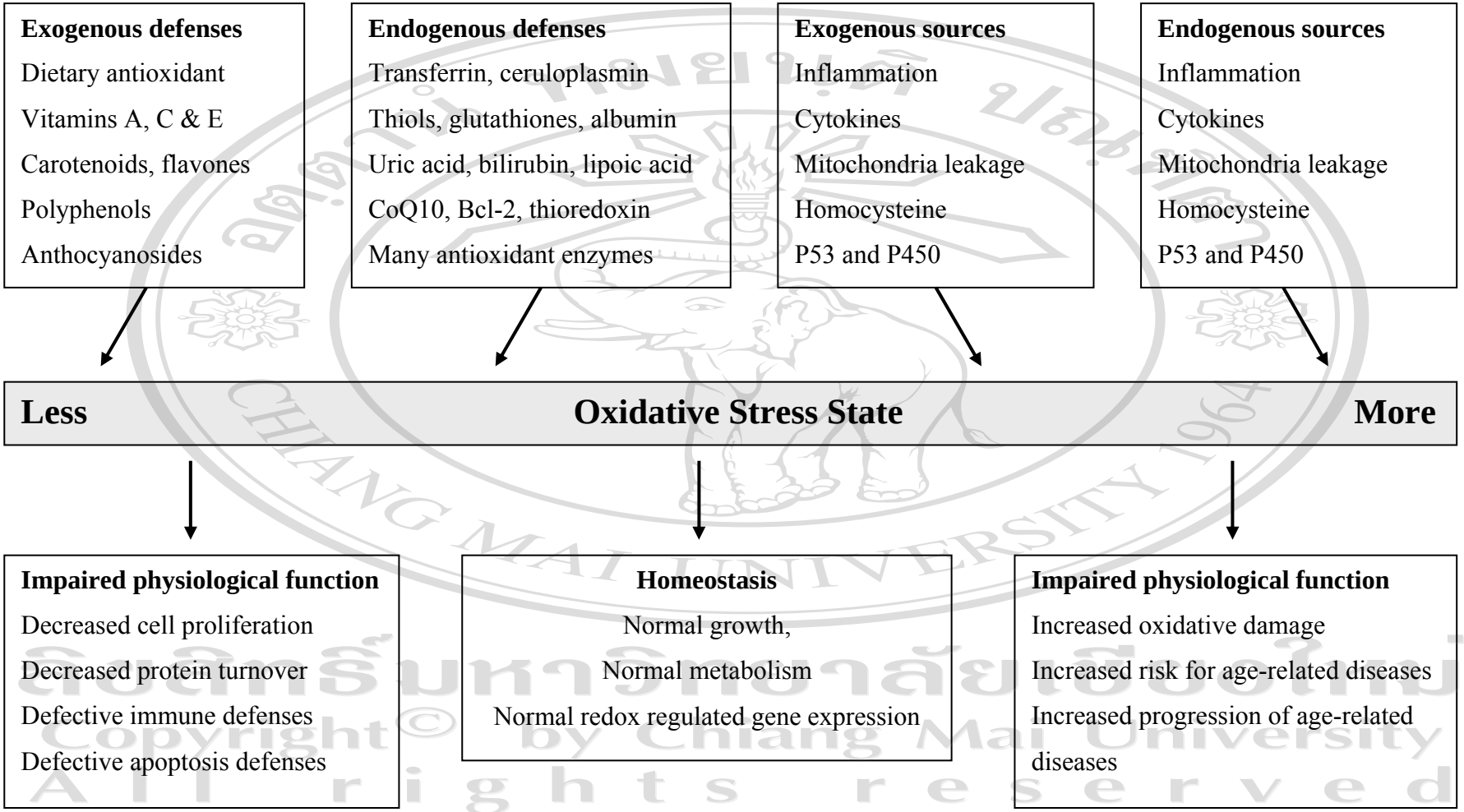
radical and a molecule- which must then result in a radical since the total number of electrons is odd), by cleavage of a radical to give another radical and, finally via redox reactions. Radicals are generally less stable than non-radical species, although their reactivity varies (Table 2.3) [10, 111-116]. In the present, oxidation reactions are of major significance in human physiology as well as the food industry. There has long been concerned with this issue. There has been an increased interest among researchers in the role of antioxidants in the diet and their impact on human health.

Table 2.3 Characteristics of ROS and RNS [10, 112]

Name	formula	Radical (R) or non-radical (NR)
Nitric oxide	$NO^{\bullet}$	R
Superoxide	$O_2^{\bullet-}$	R
Peroxyl	$ROO^{\bullet}$	R
Hydrogen peroxide	H_2O_2	NR
Hydroxyl	$OH^{\bullet}$	R
Peroxynitrite	$ONOO^-$	R
Hypochlorous acid	HOCl	NR

2.2.1 Oxidative stress and human diseases

Oxidative stress is the amount of free radical damage that is being produced in an organelle / cell / organ / organism. A free radical is a very reactive atom with an unpaired electron, which can be in a reduced or oxidized state. The majority of free radicals that damage biological systems are oxygen radicals. What determines the amount of free radicals being produced depends on the balance of many different factors, some of which are shown in Scheme 2.2 [117].



Scheme 2.2 Oxidative stress and human diseases [117].

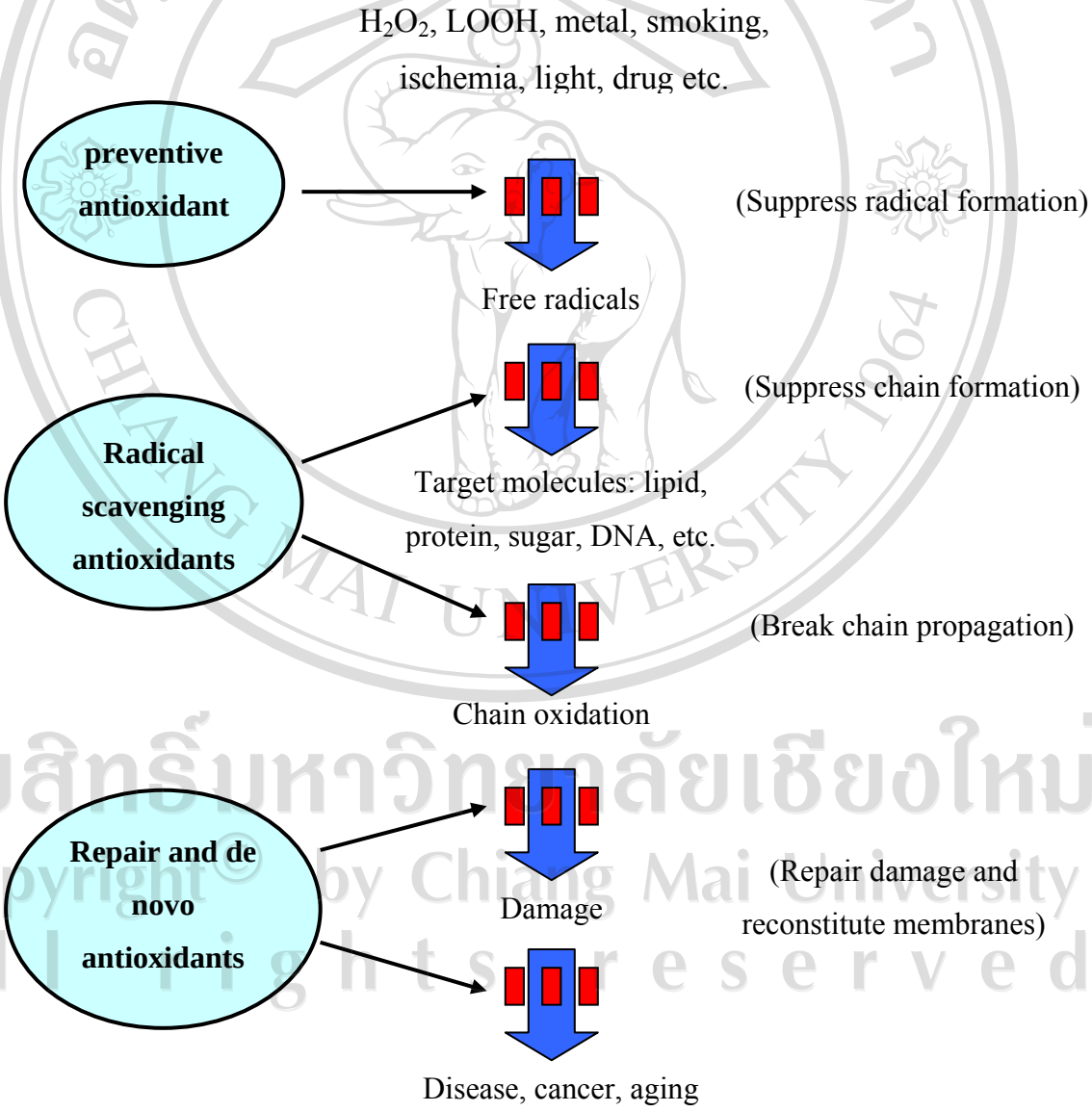
2.2.2 Oxidative stress defense systems [52, 118]

An antioxidant has been defined as "any substance that, when present at low concentration compared to a dose of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate". Antioxidants can act at different levels in the oxidative sequence. As lipid peroxidation is concerned, they could act by:

1. Decreasing localized O_2 concentrations (e.g. sealing of food stuffs under nitrogen).
2. Preventing first-chain initiation by scavenging initiating radicals such as $^{\bullet}OH$.
3. Binding metal ions in a form that will not generate such initiating species as $^{\bullet}OH$, ferryl, or Fe^{3+} / Fe^{2+} / O_2 and / or will not decompose lipid peroxides to peroxyl or alkoxyl radicals.
4. Decomposing peroxide by converting them to non-radical products, such as alcohol.
5. Chain-breaking, i.e. scavenging intermediate radicals such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction. Chain-breakers are often phenols and amines.

Antioxidants acting by mechanisms 1, 2 and 3 can be called preventive antioxidants. Those acting by mechanism 3 are not usually consumed in the course of the reactions. Antioxidants of the fourth type are also preventive antioxidants, but they may or may not be consumed during the reaction,

depending on their chemical behavior (e.g. gluthathione peroxidase acts by this mechanism and being an enzyme, is a catalyst and is not consumed). Chain-breaking antioxidants, acting by combining with the intermediate radicals, will be consumed, as will antioxidants of type 2. It should be stressed that many antioxidants have multiple mechanisms of action (Scheme 2.3 and Table 2.4).



Scheme 2.3 Defense systems *in vivo* against oxidative damage [107, 118]

Table 2.4 Defenses systems against oxidative damage [111, 118]

Preventive antioxidants: suppress the formation of free radicals

1. Radical-scavenging antioxidants: Scavenge radicals to inhibit chain initiation and break chain propagation

   Hydrophilic: vitamin C, uric acid, bilirubin, albumin

   Lipophilic: vitamin E, ubiquinol, , polyphenols, carotenoids

2. Repair and *de novo* enzymes: repair the damage and reconstitute membranes lipase, protease, DNA repair enzymes, transferase

3. Adaptation: generate appropriate antioxidant enzymes and transferase them to the right site at the right time and in the right concentration

   (a) Non-radical decomposition of hydroperoxides and hydrogen peroxide

Catalase	Decomposition of hydrogen peroxide $2H_2O_2 \longrightarrow 2H_2O + O_2$
Glutathione peroxidase(cellular)	Decomposition of hydrogen peroxide and fatty acid hydroperoxide $H_2O_2+2GSH \longrightarrow 2H_2O+2GSSG$ $LOOH+2GSH \longrightarrow LOH+H_2O+GSSG$
Glutathione peroxidase (plasma)	Decomposition of hydrogen peroxide and phospholipid hydroperoxides $PLOH+H_2O+GSSG \longrightarrow LOOH+2GSH$
Phospholipid hydroperoxide	Decomposition of phospholipid

Preventive antioxidants: suppress the formation of free radicals (continued)

Glutathione peroxidase	Hydroperoxide
Peroxidase	Decomposition of hydrogen peroxide and lipid hydroperoxide
	$LOOH+AH_2 \longrightarrow LOH+H_2O+A$
	$H_2O+ AH_2 \longrightarrow 2H_2O+A$
Glutathione-S-transferese	Decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation	
Tranferrin, lactoferrin	Sequestration of iron
Haptoglobin	Sequestration of hemoglobin
Hemopexin	Stabilization of heme
Ceruloplasmin, albumin	Sequestration of copper
(c) Quenching of active oxygens	
Superoxide dismutase (SOD)	Disproportionation of superoxide
	$2O_2^{\bullet-}+2H^+ \longrightarrow H_2O_2+O_2$
Carotenoids	Quenching of singlet oxygen

All aerobic organisms, including human beings, utilize a series of primary antioxidant defenses in an aim to protect against oxidant damage, and numerous damage removal and repair enzymes to remove and/or repair

molecules that do get damaged. Those antioxidants concentrate on the non-enzymic and enzymic primary antioxidant defenses.

Antioxidants include vitamin C, which acts as a cytosolic antioxidant; vitamin E acts as a membrane antioxidant; and glutathione, which acts to protect both cytosol and membranes against free radical attacks. Also present are the glutathione-dependent enzymes, glutathione peroxidase (GSH-Px), glutathione reductase, glutathione transferase and catalase, which break down H_2O_2 to oxygen and water, and enzyme superoxide dismutase (SOD), which converts $O_2^{\bullet -}$ into H_2O_2. Some enzymes exist in several different forms. Membrane, cytosolic, and plasma forms of GSH-Px have been reported. Similarly, there are mitochondrial, cytosolic, and extracellular forms of SOD. Other important antioxidants include dietary antioxidants, polyphenols, carotenoid, ubiquinones, bilirubin and uric acid.

2.3 **Diabetes** [119]

Diabetes mellitus is a group of metabolic diseases characterized by an elevated blood glucose level resulting from defects in insulin secretion, insulin action or both. Diabetes is a chronic illness that requires continuing medical care and patient self-management education to prevent acute complications and to reduce the risk of long-term complications. Diabetes care is complex and requires that many issues, beyond glycemic control, be addressed. A large body

of evidence exists that supports a range of interventions to improve diabetes outcomes. These standards of care are intended to provide clinicians, patients, researchers, payors, and other interested individuals with the components of diabetes care, treatment goals, and tools to evaluate the quality of care. While individual preferences, comorbidities, and other patient factors may require modification of goals, targets that are desirable for most patients with diabetes are provided. These standards are not intended to preclude more extensive evaluation and management of the patient by other specialists as needed. For more detailed information, refer to references.

2.3.1 Classification and diagnosis

In 1997, the ADA issued new diagnostic and classification criteria [120]; in 2003, modifications were made regarding the diagnosis of impaired fasting glucose [121]. The classification of diabetes includes four clinical classes:

1. Type 1 diabetes (results from β-cell destruction, usually leading to absolute insulin deficiency)
2. Type 2 diabetes (results from a progressive insulin secretory defect on the background of insulin resistance)
3. Other specific types of diabetes due to other causes, e.g., genetic defects in β-cell function, genetic defects in insulin

action, diseases of the exocrine pancreas (such as cystic fibrosis), and drug- or chemical-induced (such as in the treatment of AIDS or after organ transplantation)

4. Gestational diabetes mellitus (GDM) (diabetes diagnosed during pregnancy)

Some patients cannot be clearly classified as type 1 or type 2 diabetes. Clinical presentation and disease progression vary considerably in both types of diabetes. Occasionally, patients who otherwise have type 2 diabetes may present with ketoacidosis. Similarly, patients with type 1 may have a late onset and slow (but relentless) progression of disease despite having features of autoimmune disease. Such difficulties in diagnosis may occur in children, adolescents, and adults. The true diagnosis may become more obvious over time.

2.3.2 Criteria for the diagnosis of diabetes

1. FPG ≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.*

2. Symptoms of hyperglycemia and a casual plasma glucose ≥200 mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of hyperglycemia include polyuria, polydipsia, and unexplained weight loss.

3. 2-h plasma glucose ≥200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

* In the absence of unequivocal hyperglycemia, these criteria should be confirmed by repeat testing on a different day.

2.3.3 Criteria for testing for pre-diabetes and diabetes in asymptomatic adult individuals

1) Testing should be considered in all adults who are overweight (BMI ≥ 25 kg/m^{2*}) and have additional risk factors:

1. Physical inactivity
2. First-degree relative with diabetes
3. Members of a high-risk ethnic population (e.g., African American, Latino, Native American, Asian American, and Pacific Islander)
4. Women who delivered a baby weighing >9 lb or were diagnosed with GDM
5. Hypertension (≥140/90 mmHg or on therapy for hypertension)
6. HDL cholesterol level <35 mg/dl (0.90 mmol/l) and/or a triglyceride level >250 mg/dl (2.82 mmol/l)

7. Women with polycystic ovarian syndrome (PCOS)
8. IGT or IFG on previous testing.
9. Other clinical conditions associated with insulin resistance (e.g., severe obesity and acanthosis nigricans)
10. History of CVD or other clinical conditions associated with insulin resistance (e.g., severe obesity and acanthosis nigricans)

2) In the absence of the above criteria, testing for pre-diabetes and diabetes should begin at age 45 years

3) If results are normal, testing should be repeated at least at 3-year intervals, with consideration of more frequent testing depending on initial results and risk status

*At-risk BMI may be lower in some ethnic groups.

2.3.4 Components of the comprehensive diabetes evaluation Medical history

1. Age and characteristics of onset of diabetes (e.g., DKA, asymptomatic laboratory finding)
2. Eating patterns, nutritional status, and weight history; growth and development in children and adolescents
3. Diabetes education history

4. Review of previous treatment regimens and response to therapy (A1C records)
5. Current treatment of diabetes, including medications, meal plan, physical activity patterns, and results of glucose monitoring and patient's use of data
6. DKA frequency, severity, and cause
7. Hypoglycemic episodes
8. Hypoglycemia awareness
9. Any severe hypoglycemia: frequency and cause
10. History of diabetes-related complications

- Microvascular: retinopathy, nephropathy, neuropathy (sensory, including history of foot lesions; autonomic, including sexual dysfunction and gastroparesis)
- Macrovascular: CHD, cerebrovascular disease, PAD
- Other: psychosocial problems, dental disease

Physical examination

1. Height, weight, BMI
2. Blood pressure determination, including orthostatic measurements when indicated
3. Fundoscopic examination
4. Thyroid palpation
5. Skin examination (for acanthosis nigricans, insulin injection sites)

6. Comprehensive foot examination:

- Inspection
- Palpation of dorsalis pedis and posterior tibial pulses
- Presence/absence of patellar and Achilles reflexes
- Determination of proprioception, vibration, and monofilament
- Sensation

Laboratory evaluation

1. A1C, if results not available within past 2–3 months
2. If not performed/available within past year:
   - Fasting lipid profile, including total, LDL, HDL, cholesterol, and triglycerides
   - Liver function tests
   - Test for urine albumin excretion with spot urine albumin-to-creatinine ratio
   - Serum creatinine and calculated GFR
   - Thyroid-stimulating hormone in type 1 diabetes, dyslipidemia or women over age 50

Referrals

- Annual dilated eye exam
- Family planning for women of reproductive age
- Registered dietitian for MNT
- Diabetes self-management education

- Dental examination
- Mental health professional, if needed

2.3.5 Experimental diabetes in animal model

Primarily, experimental models of induced diabetes divide into two main classes, those in which surgical extirpation of the pancreatic mass is achieved and those in which a β-cell cytotoxin is administered to destroy the insulin-secreting cells. No oral agent has proven totally effective in this latter regard. For the investigator who prefers to work with animal models of diabetes, therefore, he has recourse to either of diabetic models (rodents) and/or genetic obese stains of mice or rats. The induction of experimental diabetes in rat using a chemical, which selectively destroy pancreatic β-cell is very convenient and simple to use. The most common substances to induce diabetes in rat are alloxan and streptozotocin. The mechanism of their action in the β-cell of the pancreas has been intensively investigated.

2.3.5.1 Streptozotocin-induced diabetes

Streptozotocin (STZ) is one of several diabetogenic agents, which produces much less toxic side effects than alloxan, and it has become the chemical of choice for the induction of diabetes mellitus in experimental animal models. In addition, STZ is also used for the treatment of pancreatic

neoplasms. STZ or 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a 2-deoxy-D-glucose derivative of *N*-methyl-*N*-nitrosourea (MUN) and synthesized by *Streptomycetes achromogenes*. It is used to induce both type 1 and type 2 diabetes mellitus. It is a broad-spectrum antibiotic which consists of a D-glycopyranose and a methylnitrosourea [122-124]. The molecular weight of STZ is approximately 265 with the empirical formula $C_8H_{15}N_3O_7$ (Figure 2.1).

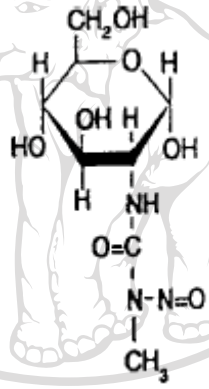


Figure 2.1 The chemical structure of streptozotocin [18].

Streptozotocin action in the β-cell is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, the hyperglycemia is observed with a concomitant drop in blood insulin. About six hours later, hypoglycemia occurs with high levels of blood insulin. Finally, hyperglycemia develops and blood insulin levels decrease [125]. These changes in blood glucose and insulin concentrations reflect abnormalities in β-cell function. STZ impairs glucose oxidation [126] and

decreases insulin biosynthesis and secretion. It was observed that STZ at first abolished the β-cell response to glucose. Temporary return of responsiveness then appears which is followed by its permanent loss and cells are damaged [125]. STZ is taken up by pancreatic β-cell via glucose transporter GLUT2. A reduced expression of GLUT2 has been found to prevent the diabetogenic action of STZ [127]. In addition, it was observed that STZ itself restricts GLUT2 expression *in vivo* and *in vitro* when administered in multiple doses [128].

The mechanisms by which STZ damages pancreatic β-cell have been proposed in Figure 2.4. Intracellular action of STZ results in changes of DNA in pancreatic β-cells, causing its fragmentation [129]. Recent studies have proved that the main reason for the STZ-induced β-cell death is alkylation of DNA [130]. The alkylating activity of STZ is related to its nitrosourea moiety, especially at the O^6 position of guanine. Damage of DNA by STZ apparently depletes NAD^+, which in turn inhibits insulin biosynthesis and leads finally to β-cell death through ATP depletion [131-132].

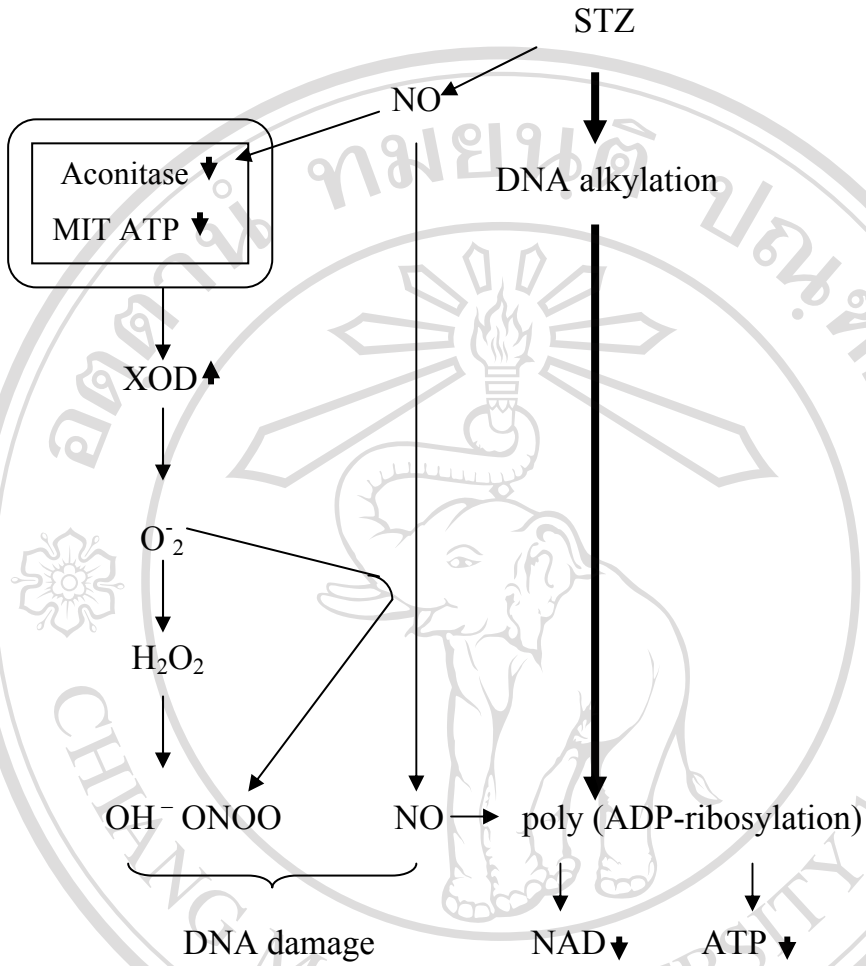
STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells. It was proposed that this molecule contributes to STZ-induced DNA damage. The participation of NO in the cytotoxic effect of STZ was confirmed in several experiments. Pancreatic β-cell exposed to STZ manifested changes characteristic for NO action, i.e. increased activity of guanylyl cyclase and enhanced formation of cGMP [133-

134]. STZ is, however, not a spontaneous nitric oxide donor. This molecule is liberatied when STZ is metabolized inside cells, but NO synthase is not required for this effect. On the other hand, the lowering of NO concentration in pancreatic islet cells by inhibition of the inducible form of nitric oxide synthase partially counteracted DNA cleavage induced by STZ [126]. A similar effect can be attained by NO scavenger [133]. However, the results of several experiments provide the evidence that NO is not the only molecule responsible for the cytotoxic effect of STZ. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells [126]. The formation of superoxide anions results from both STZ actions on mitochondria and increased activity of xanthine oxidase. It was demonstrated that STZ inhibits the Krebs cycle [134] and substantially decreases oxygen consumption by mitochondria. These effects strongly limit mitochondria ATP production and cause depletion of this nucleotide in β-cells [132]. Restriction of mitochondria ATP generation is partially mediated by NO.

Augmentation of ATP dephosphorylation increases the supply of substrate for xanthine oxidase (β-cells possess high activity of this enzyme) and enhances the production of uric acid the final product of ATP degradation. Then, xanthine oxidase catalyses a reaction in which the superoxide anion is formed from hydrogen peroxide and the hydroxyl radical is formed. The inhibition of xanthine oxidase by allopurinol restricts the cytotoxic effect of

STZ *in vitro* retreatment of β-cell with this inhibitors prevented the STZ-induced decrease of insulin secretion [132].

Thus, it can be stated that potent alkylating properties of STZ are the main reason for its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrite (ONOO; Figure 2.4) Therefore, intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.



Scheme 2.4 The mechanism of STZ-induced toxic events in β-cells of the rat pancreas [131].

STZ-induced DNA damage activates poly ADP-ribosylation. This process leads to depletion of cellular NAD^+, further reducing the ATP content [135] and subsequent inhibition of insulin synthesis and secretion [132]. The range of STZ doses is not as narrow as in the case of alloxan. The frequently used single intravenous dose in adult rats to induce type 1 diabetes is between

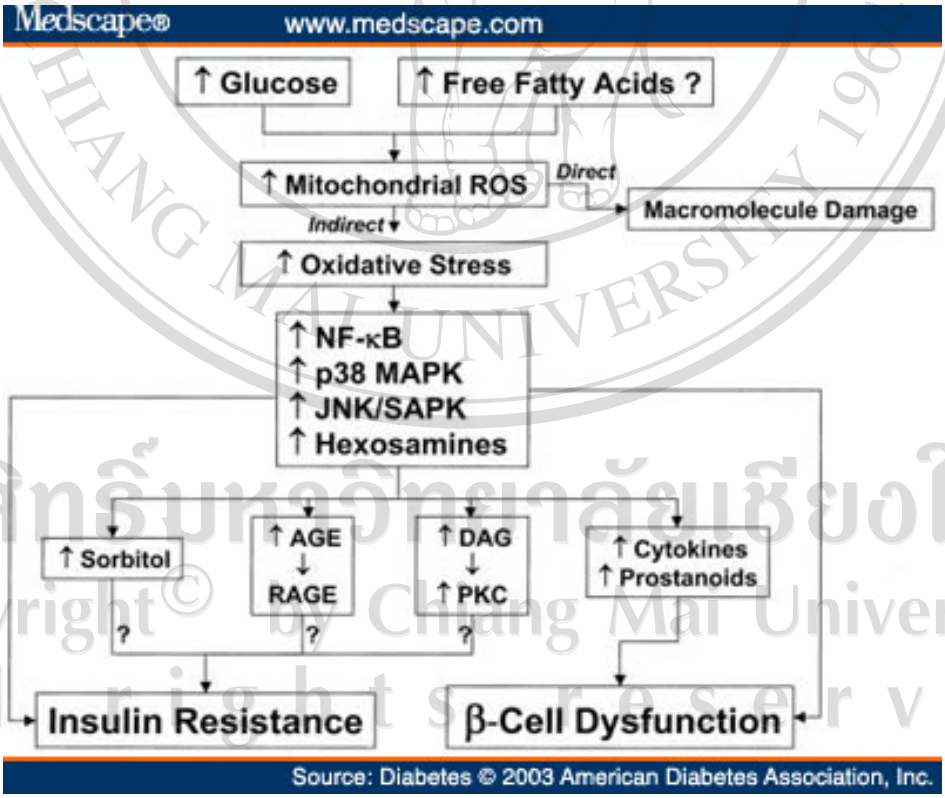
40 and 60 mg/kg BW [136], but higher doses are also used. STZ is also efficacious after intrapertioneal administration of a similar or higher dose. However, a single dose below 40 mg/kg BW may be ineffective [137].

Blondel and colleagues (1989) have developed a model of type 2 diabetes (without obesity) or previously called non-insulin-dependent diabetes in rats, obtained by injection of low dose of streptozotocin to adult male rats. Rats received streptozotocin at a dose of 45 mg/kg BW, a single intravenous injection, under light anaesthesia while they were in the non-fasting state. Diabetic rats were characterized by marked hyperglycemia, and hepatic and peripheral insulin resistance. During euglycaemic-hyperinsulinaemic clamp studies, suppression of glucose production by the liver induced by submaximal or maximal insulin levels was significantly less effective in diabetic rats as compared to normal rats. Additionally, glucose utilization was also significantly lower following both submaximal and maximal hyperinsulinaemia as compared to normal rats. Those results confirmed that insulin deficiency and concomitant hyperglycaemia, as consequences of STZ administration at a dose of 45 mg/kg BW in the adult rat, lead to the development of *in vivo* insulin resistance [138].

2.4 Oxidative stress, diabetes and antioxidants

In type 2 diabetes, additional environmental factors, including hormones, increased caloric intake, decreased physical inactivity and adiposity, have a marked influence on the disease. Now there is evidence that elevated levels of metabolic substrates contribute to the diabetic phenotype. There are considerable amounts of data indicating that the chronic elevation of plasma glucose causes many of the major complications of diabetes, including nephropathy, retinopathy, neuropathy, and macro- and microvascular damage. A role for elevated free fatty acid (FFA) levels in the development of microvascular complications remains to be established, however. Increased levels of FFAs are positively correlated with both insulin resistance and the deterioration of ß-cell function in the context of concomitant hyperglycemia. These latter effects may result from oxidative stress. There is evidence that oxidative stress, defined as a persistent imbalance between the production of highly reactive molecular species (chiefly oxygen and nitrogen) and antioxidant defenses, leads to tissue damage. Oxidative stress results from increased content of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Examples of ROS include charged species such as superoxide and the hydroxyl radicals, and uncharged species such as hydrogen peroxide. There are data indicating that ROS formation is a direct consequence of hyperglycemia. Because of their ability to directly oxidize and damage DNA, protein, and lipid, ROS are believed to play a key direct role in the

pathogenesis of late diabetic complications. In addition to their ability to directly inflict macromolecular damage, ROS can function as signaling molecules to activate a number of cellular stress-sensitive pathways that cause cellular damage, and are ultimately responsible for the late complications of diabetes. Furthermore, these same pathways are linked to insulin resistance and decreased insulin secretion. In this review, we propose that ROS and oxidative stress induced by elevations in glucose and possibly FFA levels play a key role in causing insulin resistance and ß-cell dysfunction by their ability to activate stress-sensitive signaling pathways (Scheme 2.5) [2, 9, 139-141].



Scheme 2.5 Hyperglycemia and stress-activated pathways oxidative stress [9].

Scheme 2.5 proposes a general theory of how elevated glucose and possibly FFA levels contribute to the pathophysiology of diabetes via the generation of ROS and consequent activation of numerous stress-sensitive pathways. The causative link among hyperglycemia, mitochondrial ROS generation, oxidative stress, and the development of diabetic complications has been previously suggested. ROS (and RNS), by inflicting macromolecular damage, may play a key direct role in the pathogenesis of diabetes. ROS also function as signaling molecules (analogous to second messengers) to activate several stress-sensitive pathways (indirect role). In addition, in type 2 diabetes, there is growing evidence that activation of stress-sensitive pathways, such as NF-kB, p38 MAPK, JNK/SAPK, and hexosamine, by elevations in glucose and possibly FFA levels leads to both insulin resistance and impaired insulin secretion. Thus ROS and oxidative stress, induced by elevations in glucose and possibly FFA levels, may play a key role in causing insulin resistance and ß-cell dysfunction by their ability to activate stress-sensitive signaling pathways. The proposed sequence of events may also include other stress pathways, such as the increased production of AGE, sorbitol, cytokines, and prostanoids along with PKC activation of DAG, diacylglycerol.

Oxidative stress could worsen the complications, and complications could alter requirements for antioxidant. Hyperglycemia causes oxidative stress, which increases glycosylation and oxidation of proteins involved in the pathogenesis of the complications of diabetes. Oxidative stress contributes to

impairment of islet function, insulin resistance, and microvascular and macrovascular disease. Diabetic patients with uncontrolled hyperglycemia are at risk for oxidative stress and complications, and oxidative stress may increase their requirement for vitamins with antioxidant effects. Damaged tissues may have altered responses to vitamins and differing requirements. Reduction of hyperglycemia and improvement of blood sugar control reduces oxidative stress, and reduction of free radical levels should improve metabolic function of beta cells, vascular endothelial cells, fat and muscle cells, and platelets. Decreased glycosylation and oxidation of proteins should reduce the complications such as atherosclerosis, retinopathy, nephropathy and neuropathy attributable to these processes [140-141].

Diabetes associated complications include cardiovascular diseases, nephropathy, neuropathy, retinopathy leading to blindness and embryopathy or congenital malformation (which is regarded as a type of complication of the maternal disease). Hyperglycemia is the primary clinical manifestation of diabetes and this is followed by severe ketoacidosis and a decrease in plasma pH. Several morphological alterations including fibrosis and degranulation of β-cells have been observed in the human pancreatic islets. Oxidative stress may well be the cause of this fibrosis as lipid peroxidation and certain related products can induce the overexpression of molecules involved in the process of fibrosis. Degranulation and fibrosis thus contribute to the loss of β-cell function as has been observed in patients with type 2 diabetes. Another pathological

consequence of type 2 diabetes is islet amyloidosis (the deposition of amyloid fibrils in the pancreatic cells). It may decrease islet β-cells mass, thereby reducing the capacity for insulin release. The accelerated accumulation of glycation and oxidation end products in the collagen of diabetic subjects is widely reported. Type 1 diabetes is also marked by the destruction of the pancreatic β-cells mediated by inflammatory cytokines and by free radicals and reactive oxygen/nitrogen species (ROS/RNS) generation. A free radical is any chemical species (capable of independent existence) possessing one or more unpaired electrons, an unpaired electron being one that is alone in an atomic or molecular orbital. Free radicals are formed from molecules via the breakage of a chemical bond such that each fragment keeps one electron (free radicals may also be formed by collision of the nonradical species by a reaction between a radical and a molecule-which must then result in a radical since the total number of electrons is odd), by cleavage of a radical to give another radical and, finally, via redox reactions. Radicals are generally less stable than nonradical species, although their reactivity varies (Table 2.3). Indeed several clinical disorders including rheumatoid arthritis, cancer, inflammatory bowel diseases, neurodegenerative diseases, cardiovascular problems and the process of aging are associated with chronic inflammatory reactions (Scheme 2.1). Oxidative stress may be promoted by the nonenzymatic glycation reaction. Glycation occurs in various tissues involving the production of glycated proteins, particularly Schiff's bases, Amadori products, advanced glycation end

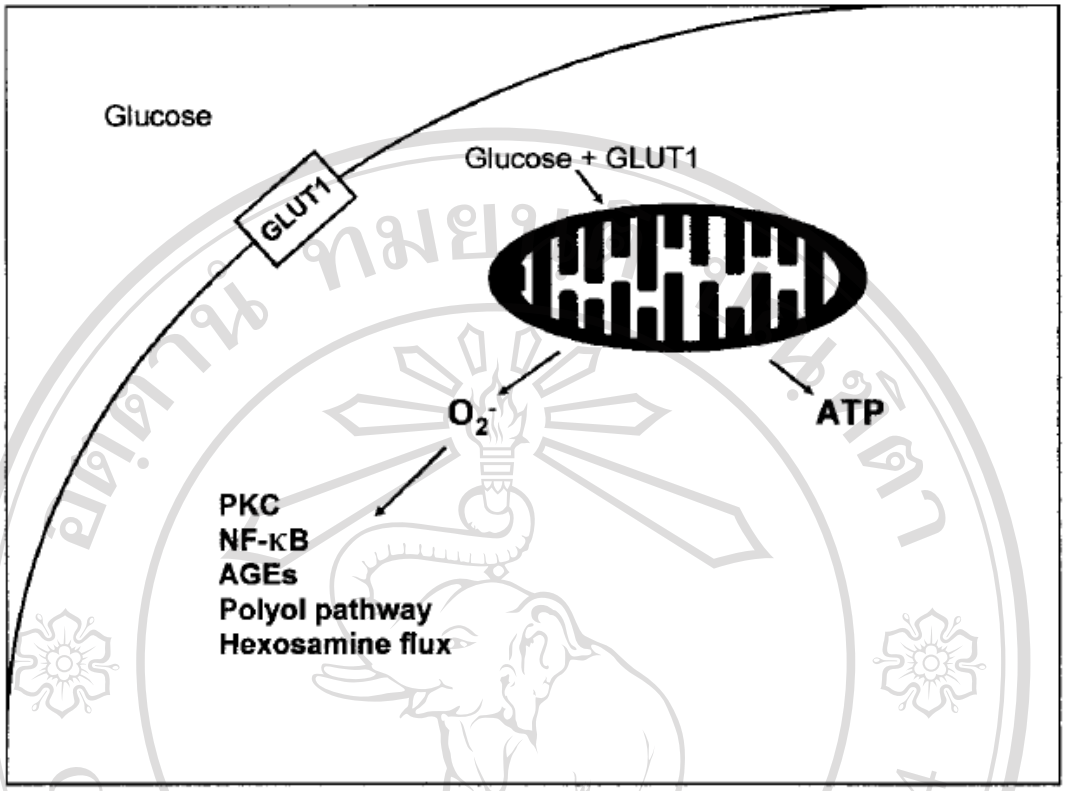
products (AGEs) and ROS. Interaction of AGEs with specific cellular binding proteins also known as the receptor for AGE (RAGE) can lead to oxidant stress, which further increases the level of ROS. Glucose autooxidation and glycoxidation could also be responsible for increased oxidative stress in diabetic individuals, indicating a complex interaction between glycation and oxidation. Glycation and cleavage of the antioxidant enzymes particularly Cu/Zn SOD can reduce their antioxidative efficacy. AGEs are suggested to bind to a cell surface receptor which results in the activation and nuclear translocation of $NF_{\kappa}B$ (that is associated with endothelial dysfunction, impaired blood flow and ischemia). Further, the level of antioxidant enzymes along with potential antioxidant vitamins is decreased in diabetic experimental animals and in humans weakening the antioxidative defense system. Evidence has shown the inactivation of glutathione synthesis in erythrocytes from type 2 diabetic patients and also a total reduction in antioxidant activity in both type 1 and type 2 diabetic patients. Long-term exposure of pancreatic β-cells to high glucose levels can cause β-cell dysfunction, while the consequent production of ROS can potentially suppress the insulin gene promoter thus impairing insulin biosynthesis and its secretion. Interestingly, the expression of GLUT 2, the glucose carrier protein, is altered in diabetic rats thus decreasing the glucose-induced insulin secretion. This could ultimately impair the insulin-stimulated glucose uptake activity contributing to an elevated glucose level [10].

The relationship between diabetes and premature vascular disease is well established. Recent prospective studies indicate that long-term glycemic control is an important predictor not only of microvascular disease, but also of macrovascular complications. Vascular endothelial cells are an important target of hyperglycemic damage, but the mechanisms underlying this damage are not fully understood. It has been suggested that, in diabetes, oxidative stress plays a key role in the pathogenesis of vascular complications, both microvascular and macrovascular, and an early marker of such damage is the development of an endothelial dysfunction. Vascular function in diabetes 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. Similarly, studies in humans with type 1 or type 2 diabetes have found endothelial dysfunction when compared with vascular function in nondiabetic subjects. In vitro, the direct role of hyperglycemia has been suggested by evidence that arteries isolated from normal animals, which are subsequently exposed to exogenous hyperglycemia, also exhibit attenuated endothelium-dependent relaxation. Consistently, in vivo studies have also demonstrated that hyperglycemia directly induces, both in diabetic and normal subjects, an endothelial dysfunction. The role of free radical generation in producing the hyperglycemia-dependent endothelial dysfunction is suggested by studies

showing that both in vitro and in vivo, the acute effects of hyperglycemia is counterbalanced by antioxidants [2, 140].

Increased superoxide production in endothelial cells during hyperglycemia: the unifying hypothesis for the development of diabetic complications. Brownlee recently pointed out the key role of superoxide production in endothelial cells at the mitochondrial level during hyperglycemia in the pathogenesis of diabetic complications. This new insight is consistent with the four pathways suggested to be involved in the development of diabetic complications (increased polyol pathway flux, increased advanced glycosylation end product formation, activation of protein kinase C, and increased hexosamine pathway flux) and with a unifying hypothesis regarding the effects of hyperglycemia on cellular dysfunction. The authors used endothelial cells subjected to physiologically relevant glucose concentrations as a model system for analyzing the vascular response to hyperglycemia because the non-insulin-dependent glucose transporter GLUT1 facilitates diffusion of high levels of glucose into the endothelium. In the presence of increased glucose, endothelial generation of reactive oxygen species, particularly superoxide anion, was shown to be enhanced. Several pathways can be considered as likely candidates for oxygen free radical formation in cells. These include NAD(P)H oxidase, the mitochondrial respiratory chain, xantine oxidase, the arachidonic cascade (lipoxygenase and cycloxygenase), and microsomal enzymes. Brownlee et al. have determined that the source of free

radicals in endothelial cells incubated in high glucose is the transport of glycolysis-derived pyruvate in mitochondria at the level of complex II (succinate:ubiquinone oxidoreductase), one of the four inner membrane–associated complexes central to oxidative phosphorylation. The data in the papers indicate that, at least in the cell culture, endothelium in an environment mimicking physiological hyperglycemia cannot control its appetite for glucose. Accelerated flux of glucose through glycolysis and feeding of pyruvate (thus formed) to the tricarboxylic acid cycle overloads mitochondria, causing excessive generation of free radicals. Although oxygen free radicals have been shown to have a physiological role in signal transduction, their sustained generation at the levels shown in endothelial cells exposed to high glucose can be expected to have substantial effects on cellular properties. Each of the pathways implicated in secondary complications of diabetes has been shown to arise by a single unifying mechanism. A central contribution of the works of Brownlee et al. is to demonstrate that suppression of intracellular free radicals, using low molecular inhibitors or by expression of the antioxidant enzyme manganese-superoxide dismutase, prevents each of these events (i.e., glucose-induced formation of oxidants is a proximal step in cell perturbation). Furthermore, activation of nuclear factor (NF)-κB by this mechanism ties hyperglycemia to the expression of multiple genes related to vascular stress response. Scheme 2.6 summarizes this finding.



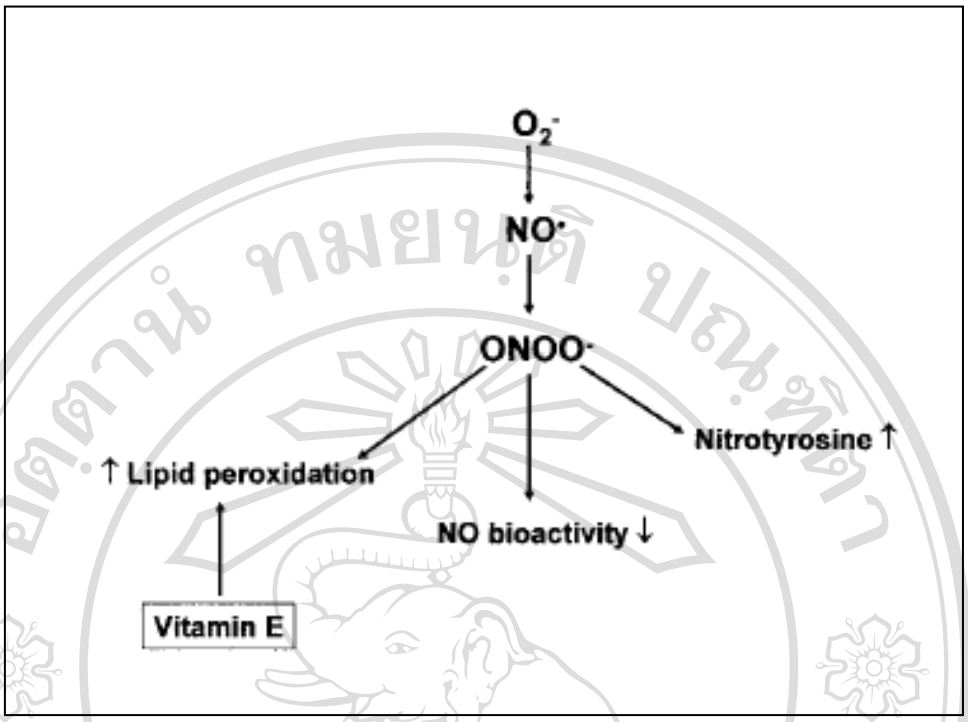
Scheme 2.6 Summarizes of mechanism of hyperglycemia to the expression of multiple genes related to vascular stress response [10]

Scheme 2.6 in endothelial cells, glucose can pass freely through the cell membrane in an insulin-independent manner via GLUT1. 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 diabetic complications, such as polyol pathway flux, increased advanced glycosylation end product (AGE) formation, activation of protein kinase C (PKC) and NF-κB, and increased hexosamine pathway flux [10].

Even increased superoxide generation in hyperglycemia is a key event in activating the other pathways involved in the pathogenesis of diabetic

complications; it represents only a first step in the production of the endothelial dysfunction in diabetes. Nitric oxide (NO) production plays a central role in modulating endothelial function. NO is generated from the metabolism of L-arginine by the enzyme nitric oxide synthase (NOS), of which there are three isoforms: the constitutive types, brain (bNOS) and endothelial (eNOS), and the inducible type, iNOS. iNOS is induced de novo by various stimuli, including hyperglycemia, while the mitochondrial-generated superoxide can inhibit eNOS, although enough NO is still produced. The superoxide anion may quench NO, thereby reducing the efficacy of a potent endothelium-derived vasodilator system that participates in the general homeostasis of the vasculature, and evidence suggests that during hyperglycemia, reduced NO availability exists. The activation of protein kinase C, due to superoxide overproduction, induces a de novo synthesis of the enzyme NAD(P)H oxidase, which significantly contributes to produce more superoxide anions. Hyperglycemia also favors, through the activation of NF-κB, an increased expression of both NAD(P)H and iNOS. Overexpression of iNOS is accompanied by increased generation of NO. Superoxide overproduction, when accompanied by increased NO generation, favors the formation of the strong oxidant peroxynitrite, which in turn avidly oxidizes tetrahydrobiopterin, an iNOS cofactor, to dihydrobiopterin. Under conditions of tetrahydrobiopterin deficiency, iNOS is in an uncoupled state, which means that electrons flowing from the iNOS reductase domain to the oxygenase domain are diverted to

molecular oxygen rather than to L-arginine, resulting in production of superoxide rather than NO. Exposure to peroxynitrite during hyperglycemia also produces an uncoupling state of eNOS, presumably via a zinc depletion of the enzyme, favoring superoxide overproduction. Consistently, in hyperglycemic conditions, an overproduction of both superoxide and NO has been reported, with a threefold increase in superoxide generation. As previously reported, the simultaneous overgeneration of NO and superoxide favors the production of a toxic reaction product, the peroxynitrite anion. The peroxynitrite anion is cytotoxic because it oxidizes sulfydryl groups in proteins, initiates lipid peroxidation, and nitrates amino acids such as tyrosine, which affects many signal transduction pathways. As previously stated, convincing evidence is now available about the possible role of oxidative stress in the development of diabetic complications. However, clinical trials with antioxidants, in particular with vitamin E, have failed to demonstrate any beneficial effect. 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 vascular oxidative stress. Scheme 2.7 helps explain this concept.



Scheme 2.7 O_2^- reacting with NO produces peroxynitrite ($ONOO^-$) [2]

Scheme 2.7 O_2^- reacting with NO produces peroxynitrite ($ONOO^-$) (see Figure 2.7 for details). The radical scavenging antioxidant vitamin E is active only against certain components of oxidative stress, particularly lipid peroxidation products, leaving other consequences of O_2^- untouched [2]. According to the evidence discussed, it is suggested that interrupting the overproduction of superoxide by the mitochondrial electron-transport chain would normalize the pathways involved in the development of diabetic complications. It might, however, be difficult to accomplish using conventional antioxidants, because these scavenge reactive oxygen species in a stechiometric manner. New low–molecular mass compounds that act as SOD or catalase

mimetics have the theoretical advantage of scavenging reactive oxygen species continuously by acting as catalysts with efficiencies approaching those of native enzymes. Such compounds normalize endothelial dysfunction in streptozotocin-induced diabetic rats and improve diabetes-induced decreases in endoneurial blood flow and motor nerve conduction velocity. Another interesting compound is L-propionyl-carnitine. This substance has been shown to act as an intracellular superoxide scavenger, improving mitochondrial function and reducing DNA damage. These properties have been shown to have beneficial effects on diabetic heart function, peripheral nerve function, and vascular blood flow in experimental diabetes [2, 4, 9].

2.4.1 Hyperglycemia and stress-activated pathways

In vivo studies have revealed that oxidative stress caused by hyperglycemia (and perhaps FFAs) occurs before the complications of diabetes become clinically evident. Wolff and Dean suggested that nonenzymatic protein glycation, a mechanism proposed early on to account for glucose cytotoxicity, was dependent on ROS (superoxide and hydroxyl) formation through transition metal-catalyzed glucose autoxidation. Research in numerous laboratories has indicated that hyperglycemia activates several major, well-characterized biochemical pathways that play a significant role in the etiology of diabetic complications. These pathways include advanced glycation end

products (AGEs) and receptors for AGE (RAGE), protein kinase C (PKC), and the polyol pathway. More recently, hyperglycemia has been implicated in the activation of additional biochemical pathways, including the stress-activated signaling pathways of nuclear factor-κB (NF-κB), NH_2-terminal Jun kinases/stress activated protein kinases (JNK/SAPK), p38 mitogen-activated protein (MAP) kinase, and hexosamine. Data now indicate that activation of these pathways is linked not only to the development of the late complications of diabetes, but also to insulin resistance and β-cell dysfunction.

2.4.2 NF-κB pathway

The most extensively studied intracellular pathway that is a target of hyperglycemia, ROS, and oxidative stress is the transcription factor NF-κB. NF-κB plays a critical role in mediating immune and inflammatory responses and apoptosis. NF-κB regulates the expression of a large number of genes, including several of those linked to the complications of diabetes (e.g., vascular endothelial growth factor [VEGF] and RAGE). Many of the gene products regulated by NF-κB in turn activate NF-κB (e.g., VEGF, RAGE), leading to a vicious circle. The aberrant regulation of NF-κB is associated with a number of chronic diseases, including diabetes and atherosclerosis. Activation of NF-κB involves the phosphorylation-induced, proteasome-mediated degradation of the inhibitory subunit, inhibitory protein κB (IκB). IκB is phosphorylated by an

upstream serine kinase, IκB kinase ß (IKK-ß), which is phosphorylated and activated by additional upstream serine kinases. A recent study in bovine endothelial cells found that exposure to hyperglycemia initially increased the production of intracellular ROS, followed by activation of NF-κB. Subsequently, PKC activity and AGE and sorbitol levels increased. Disruption of mitochondrial ROS production by several distinct approaches blocked the hyperglycemia-induced increase in ROS production. As a consequence, hyperglycemia-induced effects on NF-κB, PKC, and AGE and sorbitol levels were also suppressed. The effects of hyperglycemia on ROS formation and NF-κB activation preceded the stimulation of the other systems. Therefore, these data implicated NF-κB activation as the initial signaling event. If extended to other cell types and tissues, these findings would support the idea that ROS formation is a primary event followed by activation of the other systems.

2.4.3 JNK/SAPK pathway

The JNKs/SAPKs are members of the complex superfamily of MAP serine/threonine protein kinases. This superfamily also includes the p38 MAP kinases (p38 MAPKs) and the extracellular signal-related kinases (ERKs). In contrast to ERKs (also referred to as MAPKs), which are typically activated by mitogens, JNK/SAPK and p38 MAPK are known as stress-activated kinases, and are responsive to a variety of exogenous and endogenous stress-inducing

stimuli, including hyperglycemia, ROS, oxidative stress, osmotic stress, proinflammatory cytokines, heat shock, and ultraviolet irradiation. JNK/SAPK are activated by hyperglycemia-induced oxidative stress and are likely involved in apoptosis mediated by hyperglycemia in human endothelial cell. H_2O_2 generation, JNK/SAPK activity, and subsequent apoptosis induced by hyperglycemia could be suppressed by antioxidants such as vitamin C.

2.4.4 P38 MAPK pathway

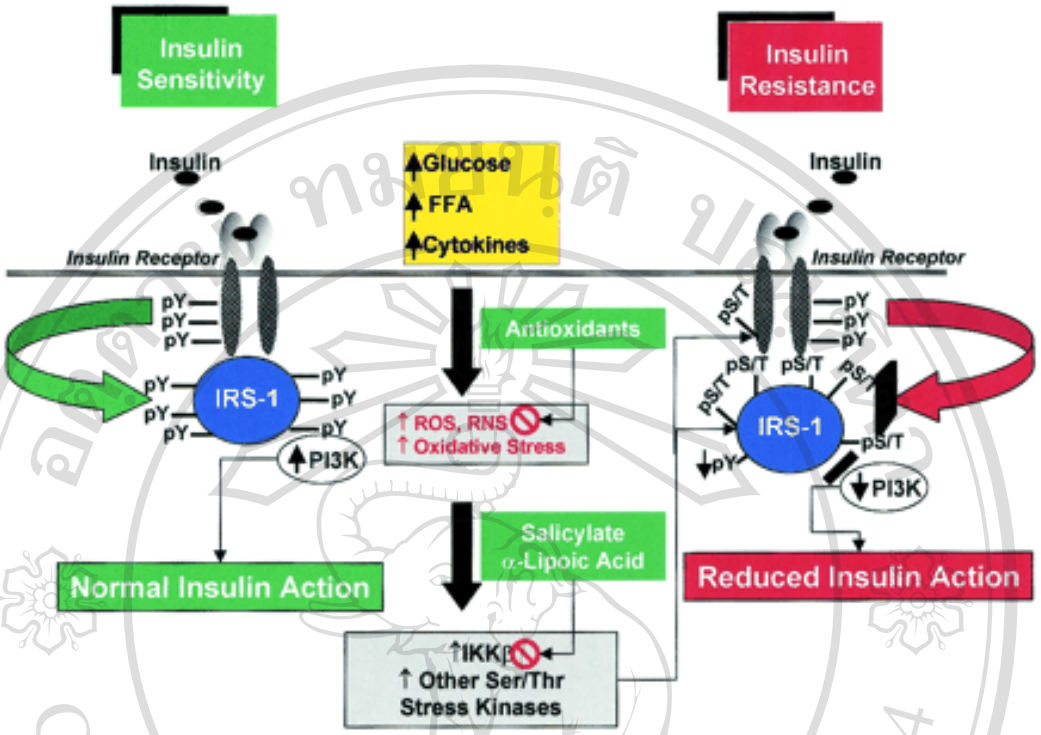
Activation of the p38 MAPK pathway occurs in response to hyperglycemia and in diabetes. In vascular smooth muscle cells, treatment with insulin and hyperglycemia induces the activation of p38 MAPK. In rat aortic smooth muscle cells, high glucose causes a fourfold increase in p38 MAPK. In a study of glomeruli of rats rendered diabetic by streptozotocin, p38 MAPK activity was increased compared with controls, followed by increased phosphorylation of heat shock protein 25, a downstream substrate of p38 MAPK. These effects were mediated by increased ROS production. Increases in total levels of JNK/SAPK and p38 MAPK have been reported in nerve tissue of patients with type 1 and type 2 diabetes, although a causative role in the pathophysiology has not been established.

2.4.5 Hexosamine pathway

The excessive flux of glucose or FFAs into a variety of cell types results in the activation of the hexosamine biosynthetic pathway, which in turn leads to insulin resistance and the development of late complications of diabetes. Recent data have implicated a hyperglycemia-induced increase in ROS formation in the activation of the hexosamine pathway. In bovine endothelial cells, hyperglycemia induced a significant increase in the hexosamine pathway, an effect that was blocked by an inhibitor of electron transport, a mitochondrial uncoupling agent (CCCP), and the expression of either uncoupling protein 1 or MnSOD. Taken together, there is strong evidence to indicate that the NF-κB, JNK/SAPK, p38 MAPK, and hexosamine pathways are stress-sensitive signaling systems that can be activated by hyperglycemia and ROS in vitro and in vivo. Chronic activation of these pathways is associated with the late complications of diabetes. This in an area worthy of continued research activity, and one that could yield new insights into the molecular pathogenesis of hyperglycemia as well as identify pharmacological targets for the treatment and/or prevention of the late complications of diabetes. What has become equally intriguing is the growing number of reports linking the activation of these same pathways to insulin resistance and β-cell dysfunction.

2.4.6 Oxidative stress relate to insulin resistance and diabetic complication

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 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 [Scheme 2.8].

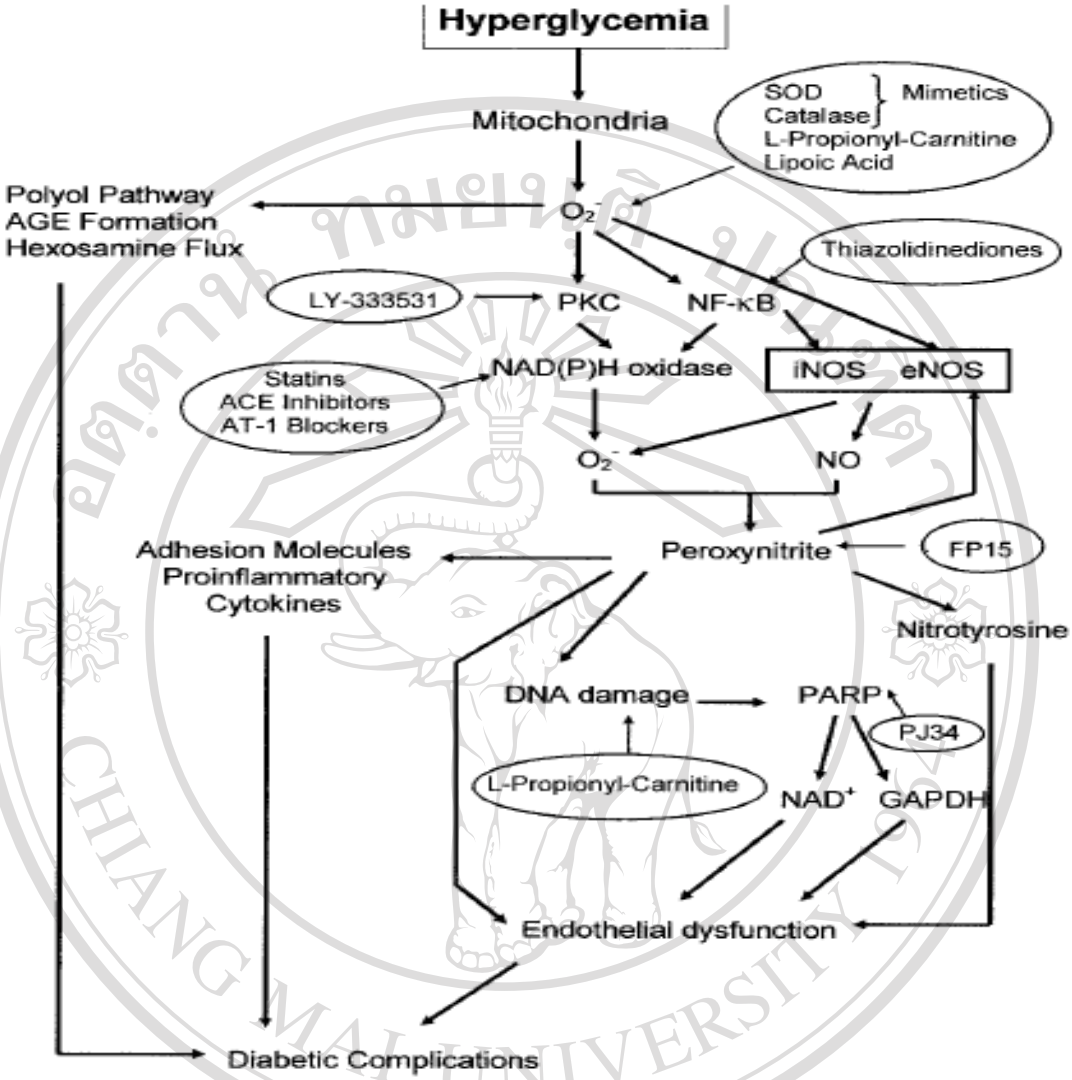


Scheme 2.8 Schematic role of serine kinase activation in oxidative stress-induced insulin resistance [9].

As shown in Scheme 2.8, 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-ß 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 [9].

Diabetic oxidative stress was generated by the role of free radical production such as superoxide and nitric oxide overproduction. It induces the hyperglycemia-dependent endothelial dysfunction that contributes to the development of diabetic complications. The acute effects of hyperglycemia are balanced by antioxidants [2, 4, 9]. Evidence exists to support the effects of antioxidants on oxidative stress induced by hyperglycemia in vitro and vivo. The effects of antioxidants, phytochemicals and traditional medicine related to oxidative stress have been studied as in Scheme 2.9



Scheme 2.9 Schematic summary of the various options proposed as causal antioxidant therapy on oxidative stress that leads to diabetic complications [2].

In Scheme 2.9 superoxide overproduction reduces eNOS activity, but through NF-κB and protein kinase C (PKC) 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 (PARP). 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. This process results in acute endothelial dysfunction in diabetic blood vessels, which contributes to the development of diabetic complications. NF-ßB activation also induces a proinflammatory conditions and adhesion molecule overexpression. All these alterations produce the final picture of diabetic complications. SOD or catalase mimetics, L-propionyl-carnitine and lipoic acid, reducing mitochondrial overproduction of superoxide, and reducing DNA damage may be good candidates for causal intracellular antioxidants. This causal therapy would also be associated with LY 333531, PJ34, and FP15, which block protein kinase ß isoform, poly (ADP-ribose) polymerase, and peroxynitrite, respectively. Other current options may be thiazolinediones, statins, ACE inhibitors, and ATI inhibitors, which also have, at different levels, intracellular causal antioxidant activity. AGE, advanced glycosylation end product.

As discussed above, the evidence suggests a role of oxidative stress in the pathogenesis of diabetic complications. This raises the concept that an antioxidant therapy may be of great interest in these patients. It has recently been suggested that diabetic subjects with complications may have a defective

cellular antioxidant response against the oxidative stress generated by hyperglycemia, which can affect to organ damage. At present, new insights into the mechanisms leading to the generation of oxidative stress are available. Most likely findings have led to the discovery and to the evaluation of new antioxidant molecules, such as SOD and catalase mimetics, that hopefully may inhibit, at an early stage, the mechanism leading to diabetic complications. While waiting for these new and specific compounds, it is reasonable to suggest using already-available synthetic substances, such as thiazolinediones, statins, ACE inhibitors, and ATI blockers [2, 9]. However, natural antioxidants from plants may also be used if they are effective causal antioxidants.

2.4.7 Biomarkers of oxidative stress in diabetes

Markers of oxidative stress and their site of location in diabetes are highlighted in Table 2.5 supporting the concept that free radicals play a key role in the pathology of diabetes. Measurement of their metabolites is considered to be a reliable marker for assaying lipid peroxidation *in vivo* and is widely recommended for studies of lipid peroxidation in human diseases and for assessment of the *in vivo* effects of antioxidants. Increased levels of TBARS (MDA), fapyadenine, fapyguanine and 5-OH uracil in the embryos and liver of diabetic mothers supported the concept of oxidative lipid and DNA damage in diabetes. Immunological studies pointed out a significantly higher

autoantibody level against MDA, 4-hydroxy-2-nonenal and ROS modified proteins which are immunogenic, indicating increase oxidative stress during diabetic conditions. Higher levels of various parameters such as plasma MDA, H_2O_2, hydroperoxides and volatile organic compounds have been reported in diabetes. This evidence, it becomes clear that exogenous antioxidants have promising effects on diabetic conditions, complementing the endogenous antioxidant defense system. A possible mechanism underlying the preservation of the β -cells is that the antioxidant suppressed apoptosis induced by ROS without affecting the cell proliferation rate [142]. Vitamin E and Vitamin C, common dietary antioxidants were demonstrated to preserve the insulin content. There is also emerging in vivo evidence which indicates that genetic enhancement of SOD activity targeted at the β-cell of the pancreas can give resistance to ROS mediated diabetogenesis. Thus transgenic mice overexpressing the radical scavenging enzyme Cu/Zn SOD in β-cells had enhanced resistance to oxidative stress-mediated diabetes. The improvement in glucose metabolism occurs in diabetic animals and diabetic humans treated with α-lipoic acid. Maddux et al. [143] have reported the beneficial effects of α-lipoic acid as a potential scavenger of H_2O_2 , safeguarding the insulin level from attack by H_2O_2 and counteracting the decrease of reduced glutathione concentration in rat muscle cells.

Table 2.5 Markers of oxidative stress and their site of location in diabetes [10].

Biological markers	Site of identification
Glucose	plasma, urine
Superoxide, Nitric oxide	plasma
Lipid peroxides, MDA	plasma, pancreatic, renal, urine
8-epi-$PGF_{2\alpha}$	plasma, urine
8-OHdG	lymphocytes, urine, pancreatic cell
8-hydroxyguanine	plasma, lymphocytes

Interestingly, polyphenolic compounds (in plants) are reported to inhibit cell hyperproliferation, tumor progression (in diabetic rats), increased vascular endothelial growth factor, decreased lipid peroxidation (MDA), superoxide radical scavenging activity by dose dependently [144]. Di Leo et al. showed that taurine and vitamin E supplementation a long with selenium reduced biochemical retinal alterations in diabetic rats. The water-soluble vitamin E analog, trolox, has been shown to partially normalize a diabetes induced change in the ratio of retinal capillary endothelial cells to pericytes [145]. Jasprica et al. conducted a study on healthy male volunteers to investigate daily intake of powdered propolis extract. After 30 days, it was found that they had decreased levels of MDA concentration which was statistically significant (p = 0.010). In addition, a 20.9 % increase in SOD activity and change in some of the red blood cell parameters were detected [146]. In addition, prevention of vascular changes in diabetic subjects by inhibiting $NO^{\bullet}$ formation occurred [147].