7

Oxidative Stress in Type II Diabetes Mellitus and the Role of the Endogenous Antioxidant Glutathione

Stephney Whillier[1], Philip William Kuchel[2] and Julia Elizabeth Raftos[1]
[1]*Macquarie University,*
[2]*University of Sydney, Sydney,*
Australia

1. Introduction

Oxidative stress appears to be involved in aging and a great many diseases, including diabetes mellitus (Lang, Naryshin et al. 1992; Fletcher and Fletcher 1994; Julius, Lang et al. 1994; Richie, Skowronski et al. 1996; Nuttall, Martin et al. 1998; Lang, Mills et al. 2000; Erden-Inal, Sunal et al. 2002; Junqueira, Barros et al. 2004; Gil, Siems et al. 2006). 'Oxidative stress' is a term that was introduced by Sies in 1985 and refers to any situation where there is a serious imbalance between the production of free radicals (FR) or reactive oxygen species (ROS), called the oxidative load, and the antioxidant defense system. The oxidative load is described as "a measure of the steady-state level of reactive oxygen or oxygen radicals in a biological system" (Baynes 1991). Oxidative stress has been defined as "a disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to potential damage" (Sies 1985). Because it is hard to measure oxidative stress directly, it is inferred from the accumulation of oxidation products, such as plasma $O_2^{\bullet-}$ radicals or high levels of peroxidation products such as thiobarbituric acid-reactive substances (TBARS) in plasma (Dominguez, Ruiz et al. 1998).

Cells can tolerate moderate oxidative loads by increasing gene expression to up-regulate their reductive defense systems and restore the oxidant/antioxidant balance. But when this increased synthesis cannot be achieved due to damage to enzymes, or substrate limitations, or when the oxidative load is overwhelming, an imbalance persists and the result is oxidative stress. Persisting imbalance leads to damage to DNA, proteins and lipids, and cell death. Oxidative stress has been implicated in over 100 diseases, more as a consequence of the pathology than as the causative factor (Halliwell 2005).

Glutathione, the tripeptide γ-L-glutamyl-L-cysteinyl-glycine (GSH), is an antioxidant molecule synthesised in almost all living cells from prokaryote organisms to the eukaryote kingdoms (Griffith and Mulcahy 1999). Glutathione is able to protect cells from oxidation by virtue of the reducing power of the thiol group on the cysteine portion of the molecule. Normally ~98% of the total GSH in healthy human cells exists in the reduced form (Griffith 1981; Kennett, Bubb et al. 2005). When GSH is oxidised to glutathione disulphide (GSSG), the enzyme GSH reductase (GR; EC 1.6.4.2) rapidly reduces it back to GSH using NADPH as an electron donor, thus ensuring that the cycling of ROS does not alter the GSH to GSSG

concentration ratio of ≥ 100 (Griffith 1999; Griffith and Mulcahy 1999). It is only when the oxidative load on the cell reaches the high levels usually associated with a variety of disease states, that a shift in this redox buffer can occur, and can result in oxidative damage to cellular constituents and the especially vulnerable plasma membrane (Kennett and Kuchel 2006).

The red blood cell (RBC) is susceptible to oxidative damage through its role as the main oxygen transporting cell, and is protected from a constant flux of reactive oxygen metabolites. Glutathione is the main source of RBC antioxidant protection and its redox status is often used as a measure of oxidative stress (Kennett and Kuchel 2006). In addition, RBC GSH has far reaching effects in being the largest mobile antioxidant pool, detoxifier and chemokine scavenger in the body (Keenoy, Vertommen et al. 2001; Sailaja, Baskar et al. 2003). Its membrane is permeable to $O_2^{\bullet-}$ and H_2O_2 (via the band 3 transporter, also called capnophorin) and so the RBC scavenges these ROS and protects cells and tissues of the body (Aoshiba, Nakajima et al. 1999).

A consistent finding in type II diabetes mellitus (non-insulin dependent diabetes mellitus, NIDDM) is a high FR load, which is associated with the hyperglycemia. The elevated glucose binds to proteins, glycating them, and leads to the formation of advanced glycation end products (AGEs) and many FRs (Keenoy, Vertommen et al. 2001). Advanced glycation end products are associated with tissue damage and aging (Sullivan 1996; Wautier and Schmidt 2004). The glycation of proteins in diabetes increases the rate of FR production by nearly 50-fold, and the generated FRs were found to increase peroxidation of polyunsaturated fatty acids (PUFAs) in the cell membrane nearly 2-fold over control levels (Mullarkey, Edelstein et al. 1990).

The less well the diabetes is controlled, i.e., the higher the blood glucose concentration, the more likely is the formation of glycated proteins. Glycated haemoglobin (HbA_{1c}), an Amadori product, is routinely measured in NIDDM, because once formed, it remains for the lifetime of the RBC (120 days), and therefore acts as a reliable monitor of blood glucose control over 4 months. Glycation of haemoglobin of under 6% is normal, but in NIDDM it can be measured in the teens. HbA_{1c} is more negatively charged than haemoglobin and has a higher oxygen affinity, therefore reducing gaseous exchange at the tissues (Arese and Schwarzer 2003).

A decrease in GSH concentration in the RBCs of NIDDM patients is widely reported, and this depletion of GSH is considered to be indicative of increased oxidative stress (Bono, Caimi.G et al. 1986; Tho, Candlish et al. 1988; Murakami, K. et al. 1989; Jain and McVie 1994; Yoshida, Hirokawa et al. 1995; Vijayalingam, Parthiban et al. 1996; Lang, Mills et al. 2000; Dincer, Akcay et al. 2002; Arese and Schwarzer 2003; Beard, Shangari et al. 2003; Martin-Gallan, Carrascosa et al. 2003; Sailaja, Baskar et al. 2003; Darmaun, Smith et al. 2005; Sampathkumar, Balasubramanyam et al. 2005). However, many authors report unchanged or elevated GSH concentrations in the RBCs of NIDDM patients (Caren and Carne 1951; Matsubara, Ferreira et al. 1992; Sinclair, Girling et al. 1992; Di Simplicio, De Giorgio et al. 1995; Laaksonen, Atalay et al. 1996; Thornalley, McLellan et al. 1996; Atalay, Laaksonen et al. 1997; Straface, Rivabene et al. 2002; Nwose, Jelinek et al. 2006; Ozkilic, Cengiz et al. 2006). Reported concentrations depend upon the form of glutathione measured (oxidised, reduced, total free, protein-bound), the analytic methods used, whether the subjects have been receiving treatment, and the particular drugs used in treating the disease (Yoshida, Hirokawa et al. 1995; Bravi, Armiento et al. 2006). Compounding this is the wide range of GSH concentrations in the healthy population, and subjects who vary in age and extent of pathology (Richie, Skowronski et al. 1996; Lang, Mills et al. 2000).

In this chapter we explored the nature of the FR, what GSH is and how it works to neutralise FRs, and how FR and AGE induced damage is implicated in the pathogenesis and complications of NIDDM. We explore the literature on the purported levels of GSH in patients with NIDDM, and finally we discuss the research done by the authors on the concentration of GSH and the rate of synthesis of GSH in a group of patients with NIDDM, compared to a group of healthy controls.

2. The nature of Free Radicals (FRs)

A FR is any species that is capable of an independent existence and is reactive because it contains at least one unpaired electron in its outer atomic orbital. The lone hydrogen atom is reactive because it has one unpaired electron. When the unpaired electron (denoted by •) resides on an oxygen atom it is called an oxygen-centred radical, and examples are the superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) radicals (Halliwell 2005). ROS are both oxygen-centred FRs as well as nonradical derivatives (e.g. H_2O_2). Radicals can react with each other to form nonradical derivatives that are often more damaging than the parent radicals (Halliwell 2005). For example $O_2^{\bullet -}$ and nitric oxide ($NO^{\bullet}$) can form the nonradical derivative peroxynitrite ($ONOO^-$). When FRs react with nonradicals, a chain reaction of FR formation can develop. One example is the reaction between $OH^{\bullet}$ and a fatty acid that produces a chain reaction of lipid peroxidation. Some FRs are beneficial to a cell; e.g. $O_2^{\bullet -}$ produced by phagocytes kills bacteria and endothelial derived $NO^{\bullet}$ has a vasodilatory function and is also produced by macrophages to kill invading microbes.

When a FR or ROS reacts with another atom or molecule, it captures one or more electrons. The FR or ROS is the electron acceptor because it will pair up its own unpaired electrons as a result of this reaction. The removal of an electron from an atom is a process called 'oxidation', thus FRs or ROS act as oxidising agents. In its broadest sense, oxidation occurs in an atom or molecule when it is involved in a reaction that increases its oxidation number; this can result from the loss of an electron or a hydrogen atom, or the addition of an oxygen atom, or it can be viewed as the relative increase in the proportion of oxygen in a molecule due to the loss of other atoms. The FR or ROS, having gained an electron is said to be reduced, and its oxidation number is decreased as a result of this reaction. Oxidation of one molecule is always accompanied by the reduction of another molecule, and so these interactions are called reduction-oxidation (redox) reactions (Hill and Kolb 2001) .

The activity of cellular antioxidants should be in balance with the oxidative load. The body has a number of antioxidant defenses that prevent the damaging effects of oxidation caused by FRs or ROS. An antioxidant is any substance, present usually in low concentrations compared to the oxidising agent, that significantly delays or prevents oxidation of other molecules by acting as a reducing agent. The most important antioxidants found in the cell are superoxide dismutase (SOD) (EC 1.15.1.1); catalase (EC 1.11.1.6); thioredoxin reductase (EC 1.8.1.9); vitamins C; vitamin E; glutathione (GSH), glutathione -*S*-transferase (GST; EC 2.5.1.18) and glutathione peroxidase (GPx; EC 1.11.1.9) (Halliwell 2005).

3. What is GSH and how does it neutralise FRs?

As noted above, GSH is synthesised in almost all living cells from prokaryote organisms to the eukaryote kingdoms (Griffith and Mulcahy 1999), which emphasises both its ancient origin and its persistent cell-protective function (see Figure 1). GSH plays a major role in

defending the cell against oxidative and nitrosative damage by reacting with ROS; and the GSH in red blood cells (RBCs) has even been proposed as an indicator of health status although concentrations have been shown to vary almost two fold even among healthy individuals (Richie, Abraham et al. 1996).

The highest concentrations of GSH in mammals are found in the liver, spleen, kidneys, pancreas, and the lens of the eye. The liver is the main site of GSH production in the body where it remains largely localised, and has the highest concentration (~10 mM) (Burk and Hill 1995), while in plasma the concentration is only ~0.90 μM (Chawla, Lewis et al. 1984; Beutler and Gelbart 1985; Yang, Chou et al. 1995). Glutathione is a major metabolite of the human RBC (Lunn, Dale et al. 1979), and has a steady-state concentration of ~2 mmol (L RBC) $^{-1}$ (~3 mM in the free water space) (Raftos, Dwarte et al. 2006). Recent evidence indicates that the RBC can export GSH at a rate of up to ~0.35 μmol (L RBC)$^{-1}$ min^{-1} and thus contribute to the extracellular GSH pool (Giustarini, Dalle-Donne et al. 2008). It is hypothesised that the rate of *de novo* synthesis of GSH in healthy RBCs balances the rate of efflux of GSH, oxidised glutathione (GSSG) and GSH-conjugates (Srivastava and Beutler 1969; Srivastava, Awasthi et al. 1976; Lunn, Dale et al. 1979; Griffith 1999), and has a turnover of 4 - 6 days (Lunn, Dale et al. 1979; Griffith 1981). There is no known mechanism for the breakdown of GSH inside the RBC, as the enzyme γ-glutamyl transpeptidase (GGTP; EC 2.3.2.2), which cleaves the γ bond in GSH in many cells of the body, is absent in the RBC (Board and Smith 1977).

Glutathione is synthesised from three amino acids: glutamate, cysteine and glycine. The synthesis of GSH was first demonstrated in extracts of pigeon liver and yeast, by Snoke and Bloch (1954) and was shown to be a process requiring two enzymes (see Figure 2) (Snoke, Yanari et al. 1953; Snoke and Bloch 1954; Snoke 1955).The condensation of glutamate and cysteine requires γ-L-glutamyl-L- cysteine synthetase now more commonly called γ-L-glutamate-L-cysteine ligase (GCL;). Addition of glycine is by glutathione synthetase (GS). γ - L-Glutamate-L-cysteine ligase is the main flux controlling enzyme, and is inhibited by GSH with a K_i of 2.3 mM in the free water of the cell (1.6 mmol (L RBC) $^{-1}$) which is within the normal range of the GSH concentration in RBCs (Richman and Meister 1975). However, 5 and 10 mM GSH, greatly in excess of normal physiological concentrations, has been shown to inhibit the activity of the enzyme by only 22 and 31% respectively (Richman and Meister 1975), suggesting that the inhibitory effect of GSH under normal conditions is not the only factor controlling GSH synthesis in RBCs. The GSH inhibition appeared to be reduced by glutamate, indicating that GSH was binding to the glutamate receptor on the enzyme (Richman and Meister 1975).

Glutamate-L-cysteine ligase is a heterodimer, composed of a heavy catalytic subunit (GCLC), and a lighter modifier subunit (GCLM). The catalytic unit can support GSH production alone, but the modifier unit increases the rate of GSH production in the RBC by increasing the K_i for GSH inhibition and decreasing the K_m for glutamate utilisation. These subunits are encoded by separate genes on different chromosomes (Griffith 1999; Yang, Dieter et al. 2002; Chen, Shertzer et al. 2005), and it may be the inherited ratio of GCLC:GCLM that determines the steady state GSH concentration of an individual. In addition, polymorphisms in both the GCLC and GCLM gene promoters have been shown to be associated with variations in the expression of these subunits, their inducibility and consequent GSH concentrations, and a number of studies have identified specific polymorphisms that increase the risk of diseases including cystic fibrosis, cardiovascular disease, schizophrenia, diabetes mellitus and asthma (Franklin, Backos et al. 2008). The

modifier subunit is not essential for viability, even though *gclm*(-/-) knockout mice have decreased levels of GSH that vary between different tissues, and their GSH concentration is substantially reduced in RBCs (Yang, Dieter et al. 2002). Both subunits can be up-regulated at the transcriptional level by oxidative stress, and especially by H_2O_2 and TNF-α (Yang, Dieter et al. 2002).

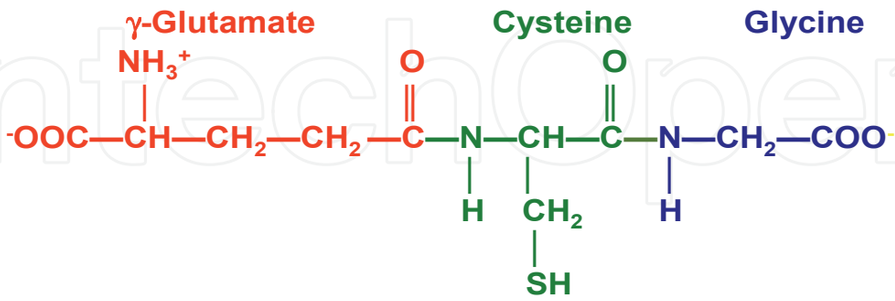


Fig. 1. **Chemical structure of GSH.** The tripeptide is composed of glutamate, cysteine and glycine residues with a γ-peptide bond between the glutamate and cysteine residues. The cysteine residue has a thiol functional group that imparts the reducing potential to the molecule.

As noted above, normally ~98% of the total GSH in healthy human cells exists in the reduced form (Griffith 1981; Kennett, Bubb et al. 2005). When GSH is oxidised to GSSG, the GR rapidly catalyses its reduction back to GSH using NADPH as an electron donor, thus ensuring that the cycling of ROS does not alter the GSH to GSSG concentration ratio of ≥ 100 (Griffith 1999; Griffith and Mulcahy 1999). It is only when the oxidative load on the cell reaches the high levels usually associated with a variety of disease states, that a shift in this redox buffer can occur, and can result in oxidative damage to cellular constituents including the especially vulnerable plasma membrane (Kennett and Kuchel 2006). A marked fall in the GSH to GSSG ratio can also act as a signal that leads to decreased cell proliferation and increased apoptosis (Schafer and Buettner 2001; Wu, Fang et al. 2004).

3.1 Free radicals and GSH in the RBC

Mammalian reticulocytes are formed in the marrow and mature into RBCs that are enucleated biconcave discs that survive for ~120 days in the circulation carrying O_2 and CO_2. Haemoglobin occupies ~33% of the RBC volume. Thus the RBC is susceptible to oxidative damage through its role as the O_2 transporter, and is protected from a constant flux of reactive O_2 metabolites. When O_2 binds to haemoglobin, iron remains in its ferrous (Fe^{2+}) form, but occasionally water or a small anion causes the transfer of an electron from the Fe^{2+} to oxygen to form methaemoglobin. Such autoxidation of haemoglobin occurs at a rate of up to 3% a day and results in the release of $O_2^{\bullet-}$ radicals (Rice-Evans and Baysal 1987; Low, Hampton et al. 2008). The release of ferric iron (Fe^{3+}) from haemoglobin leads to free radical damage by the Fenton reaction in which Fe^{3+} binding to H_2O_2 results in the formation of highly reactive $OH^{\bullet-}$ radicals. Release of Fe^{3+} can lead to oxidative changes in membrane proteins and rapid RBC aging (Burak Cimen 2008). Glutathione is the main source of RBC antioxidant protection and its redox status is often used as a measure of oxidative stress (Kennett and Kuchel 2006).

Methaemoglobin is converted back to haemoglobin by the methaemoglobin reductase system (NADH:cytochrome b_5 reductase E.C. 1.6.2.2 and cytochrome b_5: methaemoglobin reductase).

The RBC has high concentrations of SOD for the dismutation of $O_2^{\bullet-}$ to H_2O_2, making autoxidation the major source of H_2O_2 in the RBC, although H_2O_2 from extracellular sources is able to permeate RBCs, and oxidants are also generated by the interaction of xenobiotics with haemoglobin. The H_2O_2 is reduced by catalase to O_2 and H_2O, especially when concentrations of the ROS are high, and GPx, a selenium-containing enzyme, converts H_2O_2 to H_2O at lower concentrations and is the flux controlling step of GSH recycling (Low, Hampton et al. 2008). The schemes for these reactions are shown in Figure 3. Peroxide metabolism in RBCs cannot be explained by these two enzymes (catalase and GPx) alone, and kinetic modeling has shown that peroxiredoxins (Prxs), especially Prx2, which use thioredoxin as the reductant, are also important in the RBC for reducing the daily H_2O_2 load (Low, Hampton et al. 2008).

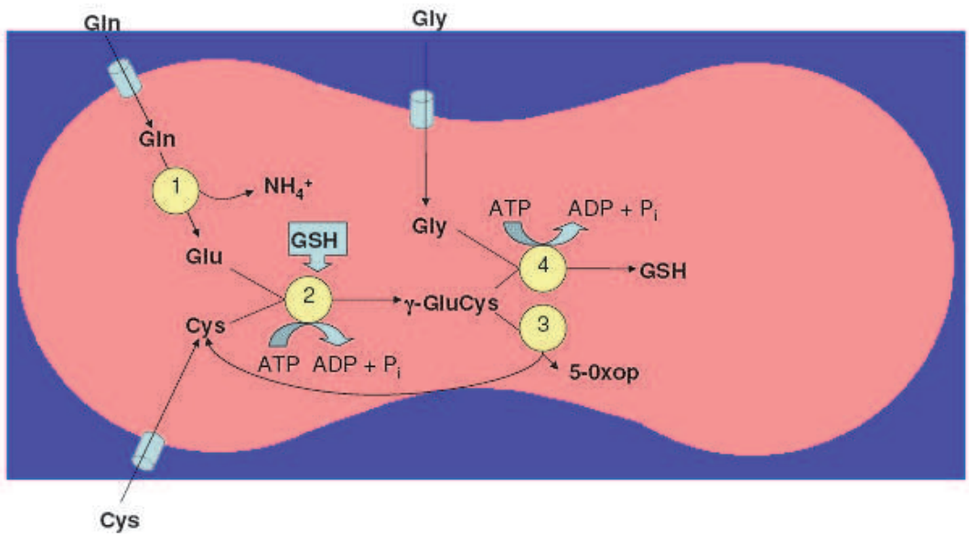


Fig. 2. **GSH synthesis in the human RBC.** The metabolite labels denote: Cys, cysteine; Gln, glutamine; Glu, glutamate; Gly, glycine; GSH, glutathione; NH_4^+, ammonium; γ-GluCys, γ-glutamylcysteine; 5-Oxop, 5-oxoproline. The enzymes are numbered as follows: (1) glutaminase (2) γ-L-glutamate-L-cysteine ligase or γ-glutamylcysteine synthetase (3) γ-glutamyl cyclotransferase (4) glutathione synthetase. (Adapted from Raftos, Whillier et al. 2007.)

Glutathione-disulphide is reduced back to GSH using GR and NADPH. The oxidative pentose phosphate pathway generates NADPH when glucose 6-phosphate is converted to 6-Phospho-glucolactone via glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49) (Mulquiney and Kuchel 2003), and this appears to be the main role of this pathway in the RBC, as the formation of ribose 5-phosphate and the other end products of the pentose phosphate pathway are of no direct synthetic use in an enucleated cell.

The reductive activity of NADPH also competes against the oxidation of PUFAs in the membrane and retains the iron in haemoglobin (Hb) in the Fe^{2+} form (Lehninger 1984). In G6PDH deficiency, there is an increased sensitivity to H_2O_2, mainly due to low NADPH concentrations. Loss of both NADPH and GSH in this inherited condition may account for enhanced methaemoglobin formation and lipid peroxidation in the propositi (Burak Cimen 2008).

Young healthy subjects have been shown to have higher GSH and lower H_2O_2 concentrations in their RBCs than healthy elderly subjects; and both have higher GSH

concentrations than elderly patients with a variety of chronic diseases (Nuttall, Martin et al. 1998). In addition, 36% of newly admitted typical hospital patients with a wide variety of chronic diseases have markedly reduced RBC total GSH concentrations compared to age-matched controls (Lang, Mills et al. 2000), which suggests that adverse changes in the redox status accompanies many chronic pathologies. There is evidence that a decrease in RBC GSH occurs in HIV, diabetes mellitus, kwashiorkor, malnutrition, magnesium-ion deficiency, alcoholic liver disease, progeria, rheumatoid arthritis, muscular dystrophy, amyotrophic lateral sclerosis, atherosclerosis, Alzheimer disease, cataractogenesis, sickle cell trait, thalassemia, and malaria (Murakami, K. et al. 1989; Sorensen, Rubin et al. 1990; Jain and McVie 1994; Becker, Leichsenring et al. 1995; Grinberg, Rachmilewitz et al. 1995; Sullivan 1996; Erden-Inal, Sunal et al. 2002; Rossi, Milzani et al. 2002).

4. How is FR and AGE damage implicated in the pathogenesis and complications of NIDDM?

Diabetes mellitus in its primary presentation is either the result of decreased pancreatic β-islet insulin production (type I or IDDM), or a resistance of cells to insulin (type II or NIDDM). The incidence of NIDDM has increased rapidly in recent years. It is estimated that the morbidities due to this illness will more than double within 15 years. Once a disease of slow-onset, appearing in middle-aged adults, it is now appearing in children (Ceriello and Motz 2004). Pathognomonic of the disease is hyperglycaemia, and this is associated with many systemic complications associated with the disease, that include pathology in the cardiovascular, neural, renal, visual and other organ systems.
A consistent finding in NIDDM is a high FR load, which is associated with hyperglycaemia. The elevated amounts of glucose lead to binding of glucose to proteins, and leads to the formation of AGEs and many FRs.

4.1 Glycation as a cause of oxidation in NIDDM

Glycation of proteins is initiated when a reducing sugar such as glucose, which in its acyclic form has an exposed aldehyde functional group, forms a double bond with the amine group of an amino acid residue (commonly lysine) in a protein (Figure 4). This is called the Maillard reaction (also called browning). The result is the formation of an imine called a Schiff base, in which the covalent bonds are rearranged to yield a ketoamine called an Amadori product. These reactions are reversible in the presence of reducing agents. Further oxidation over months results in the formation of irreversibly damaged proteins; these are the AGEs that are associated with tissue damage and aging (Sullivan 1996; Wautier and Schmidt 2004) as well as the liberation of many FRs (Keenoy, Vertommen et al. 2001). The glycation of proteins in diabetes increases the rate of FR production by nearly 50-fold; and the generated FRs increase peroxidation of PUFAs in the cell membrane nearly 2-fold over control levels (Mullarkey, Edelstein et al. 1990).
Monosaccharides become autoxidised in the presence of metal-catalysts and oxygen to yield ketoaldehydes (Wolff and Dean 1987) that then catalyse autoxidative glycosylation, in which the more reactive dicarbonyl sugars such as glucosone react with proteins to form ketoimine adducts. In turn these are more reactive than Amadori adducts and can initiate further Maillard reactions. Superoxide and H_2O_2 are formed in these autoxidation reactions (Wolff and Dean 1987). Autoxidative glycosylation therefore produces FRs, leading to damaged proteins and peroxidation of associated lipids during glycation reactions.

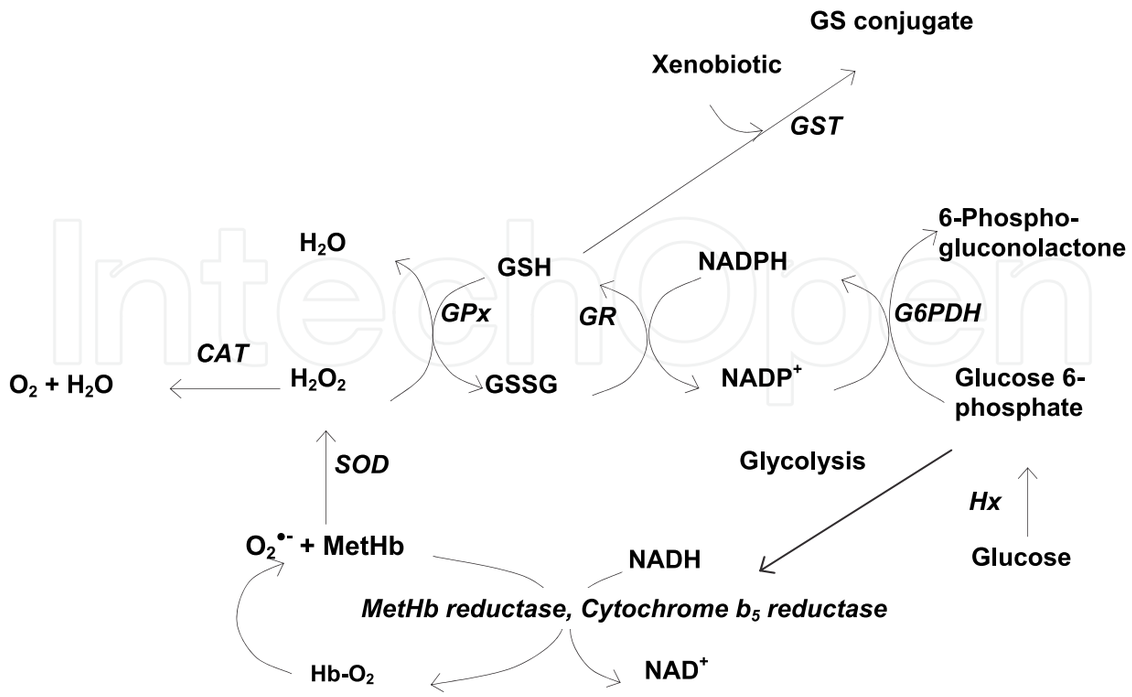


Fig. 3. **Antioxidants in the RBC.** A daily 3% formation of methaemoglobin (metHb) from oxyhaemoglobin (Hb-O_2) results in the formation $O_2^{\bullet-}$. The metHb is reduced to Hb via metHb reductase, cytochrome b_5 reductase and NADH. The $O_2^{\bullet-}$ is converted to H_2O_2 by SOD. The H_2O_2 is converted via either catalase (CAT) to O_2 + H_2O, or via GPx to H_2O, resulting in the formation of GSSG, which is reduced back to GSH using GSH-reductase (GR) and NADPH. The electron donors NADPH and NADH are derived from glycolysis and the oxidative pentose phosphate shunt respectively. The $NADP^+$ is reduced to NADPH via glucose-6-phosphate dehydrogenase (G6PDH) and glucose 6-phosphate formed via the phosphorylation of glucose using hexokinase (Hx). GSH conjugates xenobiotics which are pumped out of the RBC via ATP-dependent glutathione transferase transporters (Board 1981; Board 1993)

There are many molecules with a reactive aldehyde group that generate AGEs (Figure 5). They include acyclic fructose, formed from glucose via the sorbitol or polyol pathway; triose intermediates, such as glyceraldehyde 3-phosphate, formed in glycolysis; oxoaldehydes such as glyoxal and methylglyoxal formed from the autoxidation of these triose intermediates, the latter in a side reaction of triose phosphate isomerase (E.C. 5.3.1.1); and aldehydes such as hydroxynonenal (HNE) and malondialdehyde (MDA) formed from peroxidation of PUFAs (Dominguez, Ruiz et al. 1998; Dincer, Akcay et al. 2002; Arese and Schwarzer 2003; Beard, Shangari et al. 2003; Memisogullari, Taysi et al. 2003).

The increased polyol pathway flux only becomes significant in hyperglycaemia because the K_m for glucose is high (Brownlee 2001). In this pathway, aldose reductase (E.C. 1.1.1.21) converts carbonyl compounds like glucose to sorbitol by oxidising NADPH (Figure 5). Sorbitol can be oxidised to fructose, and NADH is generated in the process. An increase in the NADH: NAD ratio inhibits glyceraldehydes-3-phosphate dehydrogenase (GAPDH; 1.2.1.12), which requires NAD as a co-substrate. The enzyme has a thiol group at its active

site, so it is also inhibited by oxoaldehydes, glycating agents and oxidative radicals (Beisswenger, Howell et al. 2003). The consumption of NADPH decreases the ability of cells to regenerate GSH from GSSG, which elevates oxidative stress. Aldose reductase inhibition reduces diabetic neuropathy (Brownlee 2001).

The oxoaldehydes such as methylglyoxal, formed from glyceraldehyde 3-phosphate (see above), react spontaneously with GSH to form hemithioacetal. The glyoxylase pathway of two enzymes converts hemithioacetal to S-D-lactoylglutathione via glyoxylase I (E.C. 4.4.1.5), and then glyoxylase II (E.C. 3.1.2.6) converts this to D-lactate and thus regenerates GSH (Thornalley 1988; Rae, Berners-Price et al. 1990; Beard, Shangari et al. 2003). But if GSH concentrations are decreased, the tendency to form hemithioacetal, and flux via the glyoxylase pathway is diminished, and methylglyoxal will accumulate.

Methylglyoxal is increased threefold in NIDDM patients, despite significantly increased activity of the glyoxalase I enzyme and this was found to be associated with complications of the disease (Thornalley, McLellan et al. 1996). The oxoaldehyde glyoxal can also be produced from a slow non-enzymatic autoxidation of glucose (Abordo, Minhas et al. 1999).

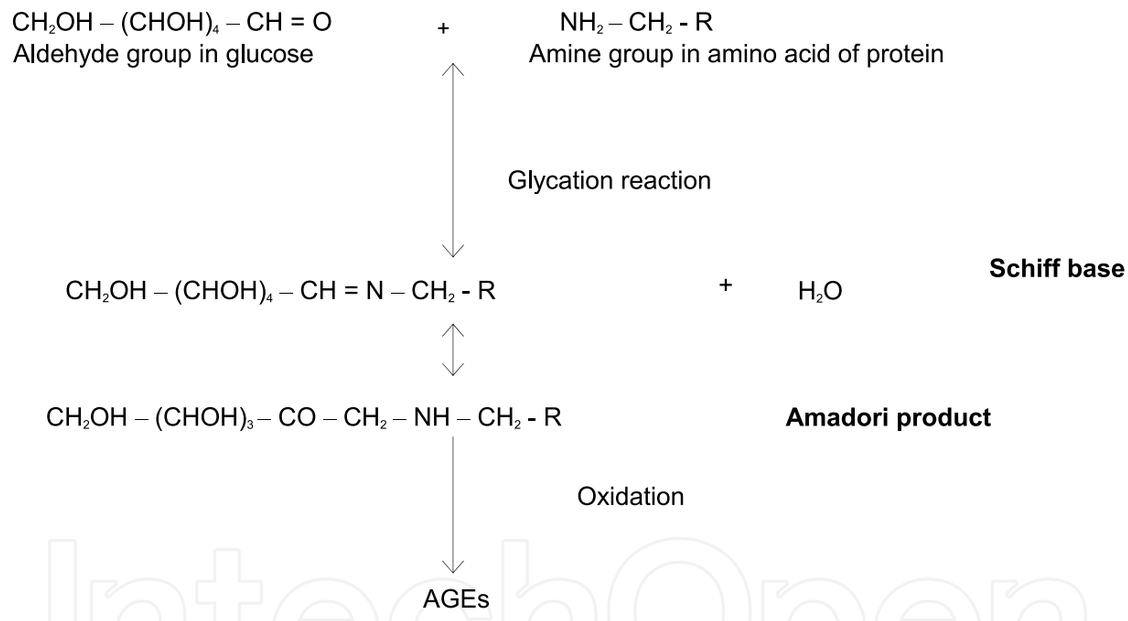


Fig. 4. **Formation of AGEs.** In the Maillard or glycation reaction, glucose forms a covalent bond with the amine group of an amino acid residue such as lysyl in a protein, resulting in the formation of an imine called a Schiff base, which has its covalent bonds rearranged into an Amadori product. Further oxidation over a time scale of months results in the formation of irreversibly damaged proteins called AGEs.

Oxidative damage to the lipids of the RBC membrane and alterations in the protein cytoskeleton result in RBC adhesion and aggregation, decreased deformability, accelerated senescence, and a reduced lifespan with increased macrophage removal (Thornalley 1988; Laaksonen, Atalay et al. 1996; Straface, Rivabene et al. 2002; Arese and Schwarzer 2003). AGEs have been identified on the RBC surface, and AGE receptors (RAGE) exist on a variety of cells, including the vascular endothelium. Binding of AGE to RAGEs on these cells leads to vascular damage and complications of cardiovascular disease (Arese and

Schwarzer 2003). This outcome is supposedly due to an inflammatory response and the generation of FRs in these cells have a disruptive effect on NO-mediated vasodilation. Superoxide in particular can directly cause vasoconstriction and decrease NO. Acute hyperglycaemia impairs endothelial cell function within 15 minutes of onset, and produces an increase in catecholamine concentrations, hence elevated mean arterial blood pressure and heart rate (Marfella, Verrazzo et al. 1995).

4.2 Oxidation in the aetiology of NIDDM

Major contributing factors to the development of NIDDM are weight gain and the deposition of excessive amounts of visceral fat (waist circumference > 94 cm in men or > 80 cm in women) (Caterson and Broom 2001). Accumulation of visceral fat is due to a reduced capacity to store excess energy subcutaneously, and is seen as a marker of 'dysfunctional adipose tissue' (Rosen and Spiegelman 2006). This fat is more lipolytic (rapidly turned over) than subcutaneous fat and less sensitive to the anti-lipolytic effect of insulin. As abdominal fat develops, the adipocytes release non-esterified fatty acids (NEFAs), many inflammatory products, and FRs (Rosen and Spiegelman 2006). Both the weight gain and inflammation are correlated with oxidative stress, which gives them an integral role in the pathogenesis as well as the complications of NIDDM.

Obesity and NIDDM are considered to be inflammatory states, and the concentration of plasma C-reactive protein, an inflammatory marker, is elevated in both conditions (Rosen and Spiegelman 2006) while macrophages are recruited to the fat deposits (Rosen and Spiegelman 2006). The potential for fat deposits to be involved in the inflammatory response may be explained by the common ontological and phylogical origin of the liver, haemopoietic and immune systems in the fat body, a structure considered to be the equivalent of mammal fat tissue. In drosophila, the fat body coordinates pathogen response with metabolic status. Specialisation occurred in the mammal, but hepatocytes are still found in close association with Kupffer cells (macrophages that are part of the immune system) with close access to blood vessels. This suggests overlapping pathways in mammals allowing for nutritionally induced inflammation (Hotamisligil 2006).

The normal physiological response to postprandial hyperglycemia is the secretion of insulin from the pancreas into the bloodstream. The hormone binds to its receptor on the cell membrane, which via its tyrosine-kinase activity initiates the phosphorylation of a tyrosine residue on an insulin receptor substrate (IRS-1). The phosphorylation stimulates a second messenger system that alters the metabolic environment in the cell, and causes the intracytoplasmic migration of vesicles that contain GLUT4 protein and their fusion into the cell's plasma membrane. GLUT4 mediates the exchange of glucose into the cell, thus rapidly lowering blood glucose concentrations to normal values within 2 h (Silverthorn 2007).

When NEFAs accumulate in cells they undergo β-oxidation, forming acetyl-CoA that enters the Krebs cycle. The excessive amount of NADH formed passes electrons into the electron transport chain, and creates a proton gradient that favours the transfer of the electrons freed from a hydrogen atom onto an oxygen molecule, forming many $O_2^{\bullet-}$ FRs. Entry of glucose into the cell increases amounts of acetyl-CoA even more; thus inhibiting the phosphorylation of tyrosine in IRS-1 as a 'protective mechanism' that down-regulates insulin sensitivity, reduces glucose entry into the cell, and thus reduces the amount of $O_2^{\bullet-}$ formed (Ceriello and Motz 2004). The NEFAs increase the concentrations of metabolites

such as diacylglycerol, fatty acyl-CoA and ceramides, and these down-regulate the number of expressed insulin receptors (Frayn 2003). Inflammatory chemicals like TNF-α also disrupt insulin action in the cell by promoting serine phosphorylation of the IRS-1, which also down-regulates the cell's sensitivity to insulin (Hotamisligil 2006).

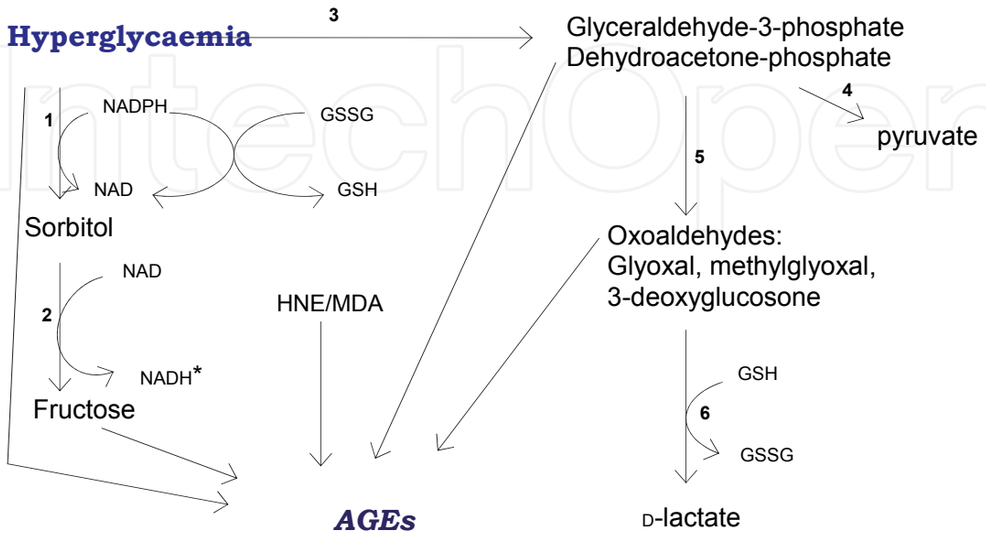


Fig. 5. **Formation of AGEs in NIDDM.** Glucose, a reducing sugar with an exposed aldehyde group in its acyclic form can directly and non-enzymatically glycate proteins and result in AGEs. It can also enter glycolysis and the triose intermediates glyceraldehydes 3-phosphate and dehydroacetone phosphate can be converted to oxoaldehydes by non-enzymatic autoxidation, particularly if GAPDH is inhibited by high levels of NADH. These oxoaldehydes can glycate proteins and result in AGE formation. Oxoaldehydes can be converted to D-lactate using 2 GSH molecules and re-releasing GSH via the glyoxylase pathway. Glucose can also enter the sorbitol, or polyol, pathway with the formation of another reducing sugar, fructose, which is more reactive than glucose in glycating proteins, and causing AGEs. The polyol pathway also depletes NADPH that is required to reduce GSSG to GSH. It results in the formation of NADH that inhibits pyruvate production and creates a favourable circumstance for more oxoaldehyde formation. Finally, aldehydes such as HNE and MDA formed from the peroxidation of PUFAs, can glycate proteins and cause AGE formation. The numbers are as follows: (1) aldose reductase (2) sorbitol dehydrogenase (3) glycolysis (4) GAPDH (5) non-enzymatic autoxidation (6) glycoxalase I and II. *NADH inhibits (4).

Randle *et al.* first showed this reciprocal interaction between the two major fuel sources (glucose and NEFAs) in 1963. It is referred to as the glucose-fatty acid cycle: high fatty acid oxidation in muscle reduces glucose uptake and oxidation i.e. insulin resistance develops (Frayn 2003). Chronically high concentrations of NEFAs released from visceral fat travel to the liver and promote hepatic insulin resistance, resulting in an impaired glucose tolerance test. Insulin resistance also results in an inability to suppress gluconeogenesis in the liver; so glucose production is increased, making the hyperglycaemia worse (Van Gaal, Mertens et al. 2006). There is also a change in activity of

lipoprotein lipase (E.C. 3.1.1.34) and triglyceride lipase (E.C. 3.1.1.3) activity in the liver, resulting in dyslipidaemias of raised total triglycerides and lowered HDL concentrations (Caterson and Broom 2001).

A vital component of the development of NIDDM is diminished pancreatic β-cell function. Many obese, insulin-resistant patients do not develop hyperglycaemia. The β-cells can increase insulin release up to fivefold, and cell volume by 50%. If blood glucose is raised artificially in healthy subjects, insulin sensitivity increases and β-cell function is enhanced within 20 h. For NIDDM to develop, the pancreas must be unable to increase insulin secretion to compensate for this insulin-resistance. By the stage that the disease is diagnosed, the β-cells are operating at 25% or less of their original capacity. The disease is progressive, and the functionality of the β-cells progressively declines (Rosen and Spiegelman 2006). Once the disease is established, hyperglycaemia has a further glucotoxic effect on the β-cells and on insulin sensitivity (Kahn, Hull et al. 2006). Non-esterified fatty acids have been shown to act directly on β-cells to regulate glucose-stimulated insulin secretion. At first they potentiate the effects of glucose, but prolonged high NEFAs inhibit insulin release (Frayn 2003).

5. Glutathione concentrations and synthesis in NIDDM; A study

Animal studies show that antioxidants such as GSH improve insulin sensitivity, and clinical trials with vitamins C and E and GSH in insulin-resistant non-diabetic subjects improves insulin sensitivity (Ceriello and Motz 2004). It has also been shown that pancreatic β-cells have few antioxidants, and therefore oxidative stress can damage mitochondria and depress insulin secretion in these cells (Ceriello and Motz 2004). However, whether the GSH concentration is altered or not in NIDDM is not clear. Quite a few authors report unchanged or elevated GSH concentrations in RBCs in diabetes (Matsubara, Ferreira et al. 1992; Sinclair, Girling et al. 1992; Di Simplicio, De Giorgio et al. 1995; Laaksonen, Atalay et al. 1996).

Glutathione is protective in oxidative stress as it forms conjugates with reactive products of lipid peroxidation such as HNE, and it participates in the conversion of methyglyoxal to D-lactate, and scavenges $O_2^{\bullet-}$ and H_2O_2 generated by AGEs (Yoshida, Hirokawa et al. 1995; Thornalley, McLellan et al. 1996; Griffith and Mulcahy 1999; Beard, Shangari et al. 2003; Beisswenger, Howell et al. 2003; Robertson, Harmon et al. 2003; Constantin, Constantinescu et al. 2005; Iles and Liu 2005; Gil, Siems et al. 2006). When loss of GSH occurs, due to an increased rate of these reactions, it is important that the metabolic machinery in RBCs react with an increased rate of GSH synthesis.

As mentioned above some authors report unchanged or elevated GSH concentrations in the RBCs of NIDDM patients (Caren and Carne 1951; Matsubara, Ferreira et al. 1992; Sinclair, Girling et al. 1992; Di Simplicio, De Giorgio et al. 1995; Laaksonen, Atalay et al. 1996; Thornalley, McLellan et al. 1996; Atalay, Laaksonen et al. 1997; Straface, Rivabene et al. 2002; Nwose, Jelinek et al. 2006; Ozkilic, Cengiz et al. 2006). A second group of authors report decreased GSH concentrations in NIDDM (Bono, Caimi.G et al. 1986; Murakami, K. et al. 1989; Yoshida, Hirokawa et al. 1995; Vijayalingam, Parthiban et al. 1996; Seghrouchni, Drai et al. 2002; Memisogullari, Taysi et al. 2003; Sailaja, Baskar et al. 2003; Constantin, Constantinescu et al. 2005; Sampathkumar, Balasubramanyam et al. 2005). It has been suggested that the GSH synthetic enzymes are glycated when glucose levels are raised, and this affects their function (Dincer, Akcay et al. 2002).

Where a decrease in GSH concentration is reported it is considered to be indicative of increased oxidative stress (Bono, Caimi.G et al. 1986; Tho, Candlish et al. 1988; Murakami, K. et al. 1989; Jain and McVie 1994; Yoshida, Hirokawa et al. 1995; Vijayalingam, Parthiban et al. 1996; Lang, Mills et al. 2000; Dincer, Akcay et al. 2002; Arese and Schwarzer 2003; Beard, Shangari et al. 2003; Martin-Gallan, Carrascosa et al. 2003; Sailaja, Baskar et al. 2003; Darmaun, Smith et al. 2005; Sampathkumar, Balasubramanyam et al. 2005).

In order to clarify this, a study was conducted to measure the GSH concentrations and rates of synthesis in the intact RBCs of 20 NIDDM patients (58.3 ± 2.5 y) with a mean HbA_{1c} of 8.34 ± 0.2%, and 20 healthy controls (ND group) (46.6 ± 3.3 y) with a mean HbA_{1c} of 5.7 ± 0.1%. Ten NIDDM subjects were being treated with both insulin and oral hypoglycemics, four were receiving insulin alone and six were receiving oral hypoglycemics alone (Whillier, Raftos et al. 2008). Ethics approval was obtained from Sydney South West Area Health Service, and the Macquarie University Human Ethics Committee.

Subjects were not fasted prior to sampling their blood. Twelve to 18 mL of blood was drawn into 6 mL lithium heparin vacutainers using a 23-gauge needle/butterfly needle and barrel. The blood was separated by centrifugation (20 min at 4˚C and 3000 × g). The plasma buffy coat (white cell layer) was aspirated and the packed cells were resuspended in 45 mL of washing solution and washed three times. A small sample of blood was used to measure the HbA_{1c} using the Micromat TM II HbA_{1c} analyser (280-0016EX) from BioRad Laboratories Pty Ltd (Regents Park, NSW, Australia). The RBCs were then resuspended to a haematocrit (Hct) of 10% in incubation solution. Samples of an Hct = 10% suspension were retained to measure the original GSH concentration of the donor's RBCs.

The Hct = 10% suspensions of intact RBCs were GSH-depleted using 1-chloro-2,4-dinitrobenzene (CDNB) (Raftos, Dwarte et al. 2006), and then incubated in a solution containing substrates for GSH synthesis (alanine, α-ketoglutarate, glycine and *N*-acetyl cysteine) and sampled for total free GSH (TFG) using a DTNB/enzymatic recycling micro-method developed by Raftos *et al* (Raftos, Dwarte et al. 2006), based on the earlier procedures of 5,5'-γ-dithiobis-(2-nitrobenzoic acid) (DTNB)/enzymatic recycling by Tietze (Tietze 1969), Owens and Belcher (Owens and Belcher 1965) and Richie *et al.* (Richie, Skowronski et al. 1996). The rate of GSH synthesis directly correlates with the rate of increase in TFG. Total RBC thiols were measured by Beutler's DTNB-based method (Beutler 1975). Plates were read on a multiskan spectrophotometer (Labsystems Original Multiscan from Pathtech Pty Ltd, Chatswood, NSW 2067).

The comparative statistics of the ND and NIDDM subjects are shown in Table 1 below. When the RBCs from control and NIDDM subjects were compared, no differences were found in their TFG concentration and in the uninhibited rate of *de novo* TFG synthesis after GSH depletion ($P > 0.05$). The NIDDM and ND control groups were also analysed for differences in TFG concentration between males and females, both between and within the groups. No differences were found ($P > 0.05$).

In subjects with NIDDM, there was a significant increase in TFG concentration with increasing age ($P = 0.0001$ for slope, ANCOVA for TFG, $F_{(1,38)} = 7.5624$, $P = 0.0093$), and a trend towards an increasing rate of TFG synthesis with age ($P = 0.09$ for slope, ANCOVA for the rate of synthesis, $F_{(1,38)} = 1.4826$, $P = 0.2313$). Neither TFG nor TFG synthesis appeared to

be correlated with age in the ND controls (P = 0.41 for GSH concentration; P = 0.94 for TFG synthesis) (Figure 6).

Variable	ND subjects	NIDDM subjects	Comparison (P value)
Age (y)	46.6 ± 3.32	58.3 ± 2.49	P < 0.01
Duration of illness (y)		15.1 ± 2.5	
HbA_{1c} (%)	5.68 ± 0.08	8.34 ± 0.22	P < 0.005
TFG concentration [mmol (LRBC) $^{-1}$]	1.91 ± 0.09	1.89 ± 0.10	P > 0.05
Maximal rate of TFG synthesis [µmol (LRBC) $^{-1}$ min $^{-1}$]	1.06 ± 0.07	1.14 ± 0.06	P > 0.05

* All values mean ± SEM

Table 1. Comparison of ND and NIDDM subjects

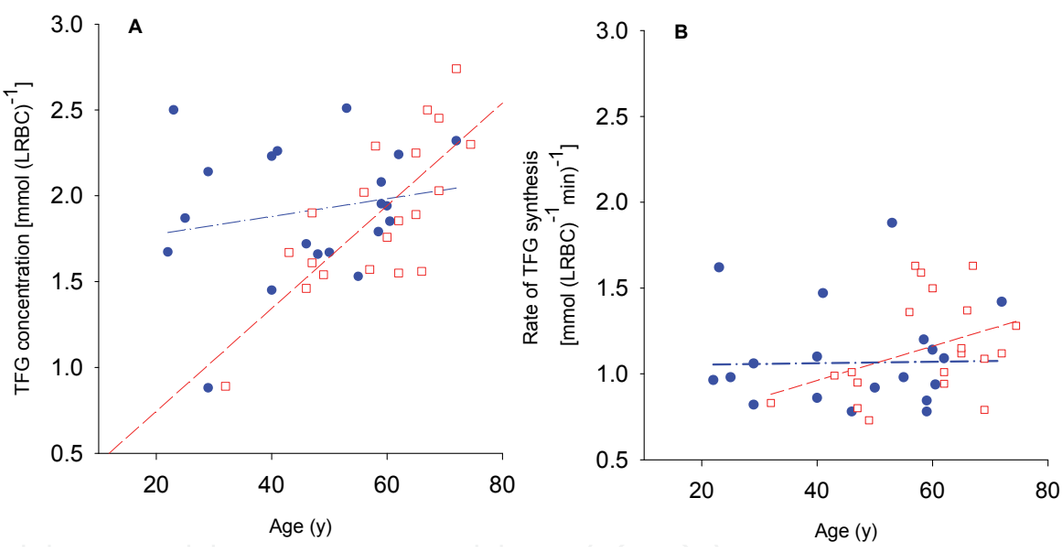


Fig. 6. **RBC GSH concentration and synthesis rate in ND and NIDDM RBCs.** A. Effect of age on TFG concentration. For ND controls blue (●), linear regression gave r = 0.20, P = 0.41; NIDDM subjects red (□), linear regression gave r = 0.76, P = 0.0001. B. Effect of age on the rate of TFG synthesis. For ND controls blue (●), linear regression gave r = 0.02, P = 0.94; NIDDM subjects red (□), linear regression gave r = 0.39, P = 0.09.

Non-diabetic subjects showed a significant correlation between the rate at which the TFG was synthesised in their RBCs, and the initial TFG concentration prior to CDNB-dependent depletion of TFG (r = 0.71, P = 0.0005). This was not the case for NIDDM subjects (r = 0.05, P = 0.731) (see Figure 7). The r and P values in Figure 7 are for the relationship between the initial TFG concentration and the rates of TFG synthesis after both variables had been corrected for the age of the subject.

In NIDDM, there was a downward trend in HbA_{1c} with age (-0.017 ± 0.02 % HbA_{1c} y $^{-1}$) (Figure 8) but the statistical analysis indicated that this was non-significant with the sample

size that was used. Therefore this was investigated in data from a further 6775 NIDDM patients where it was found to be significant with a slope of - 0.019 ± 0.002 % HbA_{1c} y^{-1} (r = 0.126, P < 0.0001). When TFG concentration and rate of TFG synthesis were corrected for age in the NIDDM group, there was no correlation between these two variables and the extent of glycation (% HbA_{1c}).

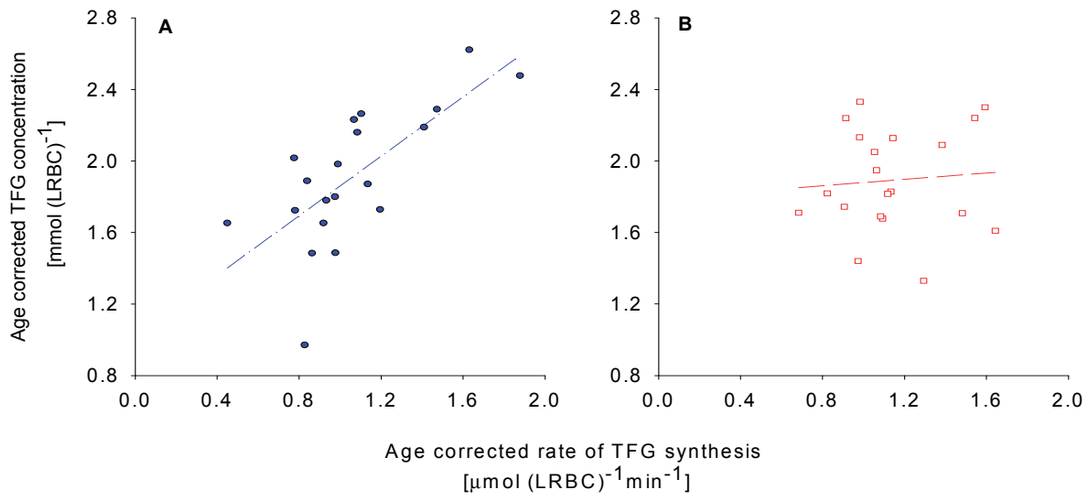


Fig. 7. **TFG synthesis rate versus concentration in ND and NIDDM A.** For ND controls (age-corrected) blue (●), linear regression gave r = 0.71 (0.69)*, P = 0.0005 (0.0006)*. B. For NIDDM subjects (age-corrected) red (□), linear regression gave r = 0.05 (0.35)*, P = 0.73 (0.14)*. (**non-age-corrected values).*

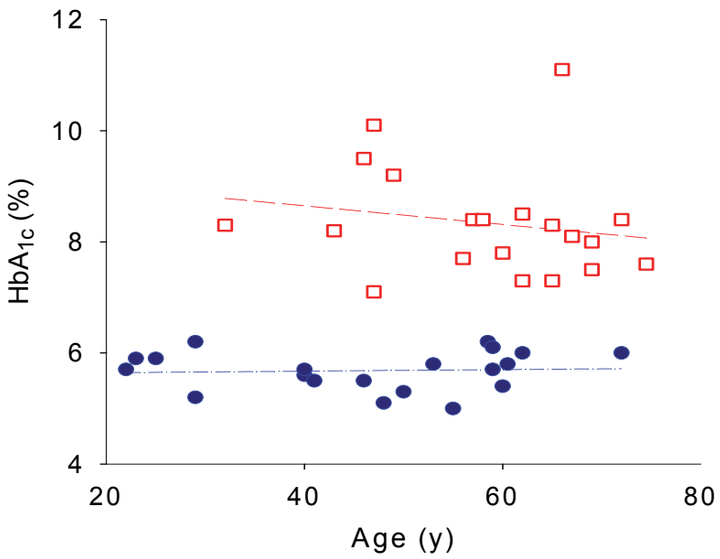


Fig. 8. **Relationship between age and Hb_{A1c}.** For ND controls blue (●), linear regression gave r = 0.06, P = 0.80. For NIDDM subjects red (□), linear regression gave r = - 0.19, P = 0.43. Additional data from 6775 NIDDM patients showed that the decline in HbA_{1c} with age was significant (slope of the linear regression -0.02 ± 0.002 % HbA_{1c} y^{-1}, r = 0.13, P < 0.0001).

5.1 Discussion of the results

Researchers are conflicted with regard to the activity of individual enzymes in the GSH synthetic pathway in RBCs in diabetic patients. Studies of purified isolated enzymes have shown decreased activity of GCL (Murakami, K. et al. 1989; Yoshida, Hirokawa et al. 1995) , GR (Murakami, K. et al. 1989; Sailaja, Baskar et al. 2003), GPx (Dincer, Akcay et al. 2002; Martin-Gallan, Carrascosa et al. 2003; Memisogullari, Taysi et al. 2003; Sailaja, Baskar et al. 2003) and GST in NIDDM (Sailaja, Baskar et al. 2003). But these findings are not supported by others (Bono, Caimi.G et al. 1986; Matsubara, Ferreira et al. 1992; Vijayalingam, Parthiban et al. 1996; Atalay, Laaksonen et al. 1997). More importantly, results obtained from individual enzyme concentrates do not necessarily reflect the overall state of GSH synthesis and utilisation in intact RBCs. The results of the present study using intact RBCs suggested that the enzymes that synthesise GSH are not damaged by glycoxidation.

When GSH-depleted RBCs are supplied with glucose and the amino acid substrates for GSH synthesis, the rate of TFG synthesis increases almost five fold compared to RBCs with normal GSH concentration (Raftos, Dwarte et al. 2006). Under these experimental conditions, the mean rate of GSH synthesis in RBCs from both NIDDM subjects and ND is the same, indicating that enzyme function had not been compromised in the NIDDM RBCs. In addition, the pre-depletion mean-TFG concentrations in RBCs in the NIDDM patients were the same as the ND subjects; this implies that the circulating RBCs of NIDDM patients are capable of increasing their rate of TFG synthesis to compensate for increased oxidative loads thought to occur in this disease.

There is evidence for increased oxidative loads in NIDDM in the present study. In the ND group there was a correlation between the rate of GSH synthesis and total GSH concentration. If the rate of oxidation were similar in healthy individuals, then the rate of synthesis in these individuals would be reflected in the GSH concentration. This was not the case in the RBCs from the NIDDM patients, and indicated that the larger variable oxidative load in NIDDM interferes with the relationship between the rate of GSH synthesis and the GSH concentration achieved in the RBC.

A novel finding in the present study was an increase in TFG concentration and to a lesser extent rate of GSH synthesis with increasing age in the RBCs from the NIDDM group but not the ND group. In addition, there was a decrease in HbA_{1c} with increasing age in the NIDDM group. This differs from reports on healthy subjects, in which GSH declined with age, and GSSG and various markers of oxidative stress, including HNE, increased (Lang, Naryshin et al. 1992; Matsubara, Ferreira et al. 1992; Gil, Siems et al. 2006). However, an increase in GSH with age in NIDDM has been shown by a few authors and has been explained variously as an up-regulation, compensation or stimulation of the GSH biosynthetic pathway by either the condition itself and/or the medication used (Matsubara, Ferreira et al. 1992; Atalay, Laaksonen et al. 1997; Nwose, Jelinek et al. 2006). In this context, the work of Matsubara *et al* is of particular interest, in showing that although the GSH concentration, GR and GPx activities do decrease with age in healthy individuals, in older NIDDM patients these values are similar to young healthy subjects. They proposed that the GSH redox system of aged subjects with diabetes is stimulated by the condition itself and/or its treatment with oral hypoglycaemics and insulin (Matsubara, Ferreira et al. 1992). Indeed, a study using *in vivo* euglycaemic hyperinsulinaemic clamping, found that administered insulin reduces RBC oxidative stress and results in a higher GSH/GSSG ratio

in NIDDM patients to almost normal levels after 2 h (Bravi, Armiento et al. 2006). The decline in HbA_{1c} seen in the NIDDM group in the present study suggests good management of the condition, and/or a decline in the oxidative load; and it may be that the treatment and management of the NIDDM patients stimulated the GSH biosynthetic pathway.

An adaptive response has been described to account for normal GSH concentrations in the RBCs of NIDDM patients. Cellular stresses such as hydrogen peroxide, TNF-α, oxidised LDL, nitric oxide and particularly HNE, are known to up-regulate GCL. Initially, GST mediates the binding of HNE with GSH to form a Michael adduct that is pumped from the cell. This results in an initial decline in GSH. But HNE and other stressors themselves increase the activity of GCL, and result in an increase in GSH. Increased GCL gene expression maintains GSH homeostasis when oxidative stress is increased (Iles and Liu 2005). GCL consists of a catalytic (GCLC) and a modulator (GCLM) subunit. It is the GCLM subunit that increases the rate of GSH synthesis by increasing the K_i for GSH inhibition and decreasing the K_m for glutamate utilisation (Yang, Dieter et al. 2002); and it has been shown that increased circulating concentrations of ROS can up-regulate the production of GCLM in certain cell types (Yang, Dieter et al. 2002).

Another possibility for the observed increase in GSH with age in the NIDDM group is that, because this was not a longitudinal study, we may have been witnessing "the survivors" in the population age-group who have genetically higher levels of GSH that actually protect them from oxidative damage. Some studies suggest this to be the case (Lang, Naryshin et al. 1992; Julius, Lang et al. 1994). Lang *et al* (1992) showed that while ~50% of healthy subjects between the ages of 60 – 79 y have low GSH concentrations, only ~25% did in the 80 – 99 y age group; so they suggested that individuals with low GSH concentrations die before reaching 80 y (Lang, Naryshin et al. 1992), and this accounts for their observed mean GSH concentrations increasing with age (Julius, Lang et al. 1994). Another study reported normal concentrations of three indicators of oxidative stress (GSH, thiobarbituric acid and diene conjugates) in elderly (>60 y) NIDDM patients (Sinclair, Girling et al. 1992). Longitudinal studies on healthy individuals and subjects with NIDDM are needed to determine whether we are observing a true maintenance in GSH concentration in NIDDM patients with age, or simply the survival of those with inherently high GSH concentrations.

6. Conclusion

Oxidative stress refers to an imbalance between oxidative load and antioxidant defenses. Type II diabetes mellitus is considered an inflammatory state with a high oxidative load. The accumulation of visceral fat results in the release of NEFAs and FR, and these are implicated in the down regulation of the insulin sensitivity of plasma membranes and in the inhibition of the release of insulin from the pancreas. The resulting hyperglycaemia causes the formation of AGEs and FR that damage endothelial linings, the kidney, nervous tissue, and the eye; and this manifests as the many complications of the disease.

Glutathione is an endogenously produced antioxidant that protects cells by virtue of the reducing power of its thiol group located on the cysteine moiety of the tripeptide. Red blood cell GSH constitutes a large mobile antioxidant pool that is able to scavenge ROS and protect tissues in the body. Glutathione is beneficial in oxidative stress in conjugating reactive products and scavenging FRs. It is important that the metabolic machinery of the RBC reacts to increase the rate of GSH synthesis when oxidative loads are high.

There is controversy in the literature on the role of GSH in NIDDM, with various authors reporting increased, unchanged, or decreased concentrations of RBC GSH. The results of the study presented here are unique in showing that the intact RBC maintains its ability to synthesise GSH at the same rate as found in healthy controls when given glucose and the amino acid substrates. This suggests that the enzymes involved in the metabolic pathway function normally. A surprising finding was an increase in TFG concentration and rate of synthesis with increasing age in NIDDM subjects but not in ND subjects; and a decrease in HbA_{1c} levels with increasing age in NIDDM subjects. It is proposed that the treatment of the condition in the form of insulin and/or oral hypoglycaemics causes an upregulation in the GSH pathway. It is also proposed that older subjects who were seen in this study are "the survivors" with genetically higher concentrations of GSH, which suggests an overall protective function of this antioxidant in this disease.

7. Acknowledgements

Many thanks to Prof. Dennis Yue, Lynda Molyneaux, Belinda Brooks and the Metabolic Unit at Royal Prince Alfred Hospital's Diabetes Centre, the doctors at the University Health Services at Macquarie University and the volunteer blood donors. BioRad Labs Pty Ltd (NSW Australia) supplied the Micromat II Alc analyser.

8. References

Abordo, E., H. Minhas, et al. (1999). Accumulation of α-oxoaldehydes during oxidative stress: a role in cytotoxicity. *Biochemical Pharmacology* 58: 641-648.

Aoshiba, K., Y. Nakajima, et al. (1999). Red blood cells inhibit apoptosis of human neutrophils. *Blood* 93(11): 4006-4010.

Arese, P. and E. Schwarzer (2003). *Metabolic Disorders: Red Cell Alterations in Diabetes Mellitus*. Berlin, Springer-Verlag.

Atalay, M., D. Laaksonen, et al. (1997). Altered antioxidant enzyme defences in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress. *Acta Physiologica Scandinavica* 161(2): 195-201.

Baynes, J. (1991). Perspectives in diabetes: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405-412.

Beard, K., N. Shangari, et al. (2003). Metabolism, not auto-oxidation, plays a role in α-oxoaldehyde and reducing sugar-induced erythrocyte GSH depletion: Relevance for diabetes mellitus. *Molecular and Cellular Biochemistry* 252: 331-338.

Becker, K., M. Leichsenring, et al. (1995). Glutathione and association antioxidant systems in protein energy malnutrition: results of a study in Nigeria. *Free Radical Biology and Medicine* 18(2): 257-263.

Beisswenger, P. J., S. Howell, et al. (2003). Glyceraldehyde-3-phosphate dehydrogenase activity as an independent modifier of methylglyoxal levels in diabetes. *Biochimica et Biophysica Acta* 1637: 98-106.

Beutler, E. (1975). *Red cell metabolism: a manual of biochemical methods*. New York, Grune & Stratton.

Beutler, E. and T. Gelbart (1985). Plasma glutathione in health and in patients with malignant disease. *Journal of laboratory & clinical medicine* 105(5): 581-584.

Board, P. (1981). Transport of glutathione S-conjugate from human erythrocytes. *FEBS Letters* 124(2): 163-165.

Board, P. (1993). Inhibition of erythrocyte glutathione conjugate transport by polyethoxylated surfactants. *FEBS* 315(3): 298-300.

Board, P. G. and J. Smith (1977). Erythrocyte γ-glutamyl transpeptidase. *Blood* 49: 667-668.

Bono, A., Caimi.G, et al. (1986). Red cell peroxide metabolism in diabetes mellitus. *Hormone and metabolic Research* 19: 264-266.

Bravi, M. C., A. Armiento, et al. (2006). Insulin decreases intracellular oxidative stress in patients with type 2 diabetes mellitus. *Metabolism: Clinical and Experimental* 55: 691-695.

Brownlee, M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813-319.

Burak Cimen, M. (2008). Free radical metabolism in human erythrocytes. *Clinica Chimica Acta* 390: 1 - 11.

Burk, R. and K. Hill (1995). Reduced glutathione release into rat plasma by extrahepatic tissue. *American Journal of Physiology* 269(3 pt 1): G396-399.

Caren, R. and H. O. Carne (1951). The blood glutathione level and its response to insulin in diabetic and non-diabetic patients and a case of insulin resistance. *The American Journal of Medical Sciences* 221: 307-313.

Caterson, I. D. and J. Broom (2001). *Obesity*. London, Harcourt Health Com.

Ceriello, A. and E. Motz (2004). Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited *Arteriosclerosis, Thrombosis, and Vascular Biology* 24: 816-823.

Chawla, R., F. Lewis, et al. (1984). Plasma cysteine, cystine, and glutathione in cirrhosis. *Gastroenterology* 87(4): 770-776.

Chen, Y., H. G. Shertzer, et al. (2005). Glutamate cysteine ligase catalysis; dependence on ATP and modifier subunit for regulation of tissue glutathione levels. *The Journal of Biological Chemistry* 280(40): 33766 - 33774.

Constantin, A., E. Constantinescu, et al. (2005). Effects of ageing on carbonyl stress and antioxidant defense in red blood cells of obese Type 2 diabetic patients. *Journal of Cellular and Molecular Medicine* 9(3): 683-691.

Darmaun, D., S. D. Smith, et al. (2005). Evidence for accelerated rates of glutathione utilization and glutathione depletion in adolescents with poorly controlled type I diabetes. *Diabetes* 54: 190-196.

Di Simplicio, P., L. A. De Giorgio, et al. (1995). Glutathione, glutathione utilizing enzymes and thioltransferase in platelets of insulin-dependent diabetic patients: relation with platelet aggregation and with microangiopathic complications. *European Journal of Clinical Investigation* 25(9): 665-669.

Dincer, Y., T. Akcay, et al. (2002). Effect of oxidative stress on glutathione pathway in red blood cells from patients with insulin-dependent diabetes mellitus. *Metabolism* 51(10): 1360-1362.

Dominguez, C., E. Ruiz, et al. (1998). Oxidative stress at onset and in early stages of type I diabetes in children and adolescents. *Diabetes Care* 21: 1736-1742.

Erden-Inal, M., E. Sunal, et al. (2002). Age-related changes in the glutathione redox system. *Cell Biochemistry and Function* 20: 61 - 66.

Fletcher, R. H. and S. W. Fletcher (1994). Glutathione and ageing: ideas and evidence. *Lancet* 344(8934): 1379 - 1380.

Franklin, C. C., D. S. Backos, et al. (2009). Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Molecular Aspects of Medicine* 30(1-2): 86-98.

Frayn, K. N. (2003). *The glucose-fatty acid cycle: a physiological perspective.* Biochemical Society, Essex, Colchester.

Gil, J., W. Siems, et al. (2006). Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes. *Free Radical Research* 40(5): 495-505.

Giustarini, D., I. Dalle-Donne, et al. (2008). Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells, Molecules and Diseases* 40(2): 174-179.

Griffith, O. W. (1981). Glutathione turnover in human erythrocytes. *Journal of Biological Chemistry* 256(10): 4900-4904.

Griffith, O. W. (1999). Biologic and pharmacologic regulation of mammalian glutathione synthesis. *Free Radical Biology and Medicine* 27(9-10): 922-935.

Griffith, O. W. and R. T. Mulcahy (1999). The enzymes of glutathione synthesis: gamma-glutamylcysteine synthetase. *Advances in Enzymology*. New York, John Wiley & sons Inc. 73: 60-80.

Grinberg, L., E. Rachmilewitz, et al. (1995). Hydroxyl radical generation in B-thalassemia. *Free Radical Biology and Medicine* 18: 611-615.

Halliwell, B. (2005). *Free Radicals and Other Reactive Species in Disease*. John Wiley & Sons.

Hill, J. W. and D. K. Kolb (2001). *Chemistry for Changing Times*. New Jersey, Prentice Hall.

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature* 444: 860-867.

Iles, K. E. and R. M. Liu (2005). Mechanisms of glutamate cysteine ligase (GCL) induction by 4-hydroxynonenal. *Free Radical Biology and Medicine* 38: 547-556.

Jain, S. and R. McVie (1994). Effect of glycemic control, race (white versus black), and duration of diabetes on reduced glutathione content in erythrocytes of diabetic patients. *Metabolism* 43: 306-309.

Julius, M., C. Lang, et al. (1994). Glutathione and morbidity in a community-based sample of elderly. *Journal of Clinical Epidemiology* 47(9): 1021-1026.

Junqueira, V. B. C., S. B. M. Barros, et al. (2004). Aging and oxidative stress. *Molecular Aspects of Medicine* 25: 5-16.

Kahn, S. E., R. L. Hull, et al. (2006). Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444: 840-846.

Keenoy, B. M., J. Vertommen, et al. (2001). Divergent effects of different oxidants on glutathione homeostasis and protein damage in erythrocytes from diabetic patients: effects of high glucose. *Molecular and Cellular Biochemistry* 225: 59-73.

Kennett, E., W. Bubb, et al. (2005). NMR studies of exchange between intra- and extracellular glutathione in human erythrocytes. *Redox Report* 10(2): 83-90.

Kennett, E. C. and P. W. Kuchel (2006). Plasma membrane oxidoreductases: effects on erythrocyte metabolism and redox homeostasis. *Antioxidants & Redox Signaling* 8(7 & 8): 1241 - 1247.

Laaksonen, D. E., M. Atalay, et al. (1996). Increased resting and exercise-induced oxidative stress in young IDDM men. *Diabetes Care* 19(6): 569-574.

Lang, C., S. Naryshin, et al. (1992). Low blood glutathione levels in healthy aging adults. *Journal of Laboratory and Clinincal Medicine* 120(5): 720-725.

Lang, C. A., B. J. Mills, et al. (2000). Blood glutathione decreases in chronic diseases. *Journal of Laboratory and Clinical Medicine* 135: 402-405.

Lang, C. A., S. Naryshin, et al. (1992). Low blood glutathione levels in healthy aging adults. *Journal of Laboratory and Clinical Medicine* 120(5): 720-725.

Lehninger, A. L. (1984). *Lehninger, Principles of biochemistry*. New York, Worth Publishers, Inc.

Low, F. M., M. B. Hampton, et al. (2008). Peroxiredoxin 2 and peroxide metabolism in the erythrocyte. *Antioxidants & Redox Signaling* 10(8): 1621-1629.

Lunn, G., G. L. Dale, et al. (1979). Transport accounts for glutathione turnover in human erythrocytes. *Blood* 54(1): 238-244.

Marfella, R., G. Verrazzo, et al. (1995). Glutathione reverses systemic hemodynamic changes induced by acute hyperglycemia in healthy subjects. *American Journal of Physiology* 268(31): E1167-1175.

Martin-Gallan, P., A. Carrascosa, et al. (2003). Biomarkers of diabetes-associated oxidative stress and antioxidant status in young diabetic patients with or without subclinical complications. *Free Radical Biology and Medicine* 34(12): 1563-1574.

Matsubara, L., A. Ferreira, et al. (1992). Influence of diabetes mellitus on the glutathione redox system of human red blood cells. *Brazilian Journal of Medical and Biological Research* 25: 331-335.

Memisogullari, R., S. Taysi, et al. (2003). Antioxidant status and lipid peroxidation in type II diabetes mellitus. *Cell biochemistry and function* 21: 291-296.

Mullarkey, C. J., D. Edelstein, et al. (1990). Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochemical and Biophysical Research Communications* 173(3): 932-939.

Mulquiney, P. J. and P. W. Kuchel (2003). *Modelling Metabolism with Mathematica*. Boca Raton, Florida, CRC Press.

Murakami, K., T. K., et al. (1989). Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. *Metabolism* 38(8): 753-758.

Nuttall, S. L., U. Martin, et al. (1998). Glutathione: in sickness and in health. *Lancet* 351(9103): 645.

Nwose, E. U., H. F. Jelinek, et al. (2006). Changes in the erythrocyte glutathione concentration in the course of diabetes mellitus. *Redox Report* 11(3): 99-104.

Owens, C. and R. Belcher (1965). A colorimetric micro-method for the determination of glutathione. *Journal of Biological Chemistry* 94: 705-711.

Ozkilic, A. C., M. Cengiz, et al. (2006). The role of N-acetylcysteine treatment on anti-oxidative status in patients with type II diabetes mellitus. *Journal of Basic & Clinical Physiology & Pharmacology* 17(4): 245-254.

Rae, C., S. Berners-Price, et al. (1990). Kinetic analysis of the human erythrocyte glyoxalase system using H NMR and a computer model. *European Journal of Biochemistry* 193: 83-90.

Raftos, J., S. Whillier, et al. (2007). Kinetics of uptake and deacetylation of N-acetylcysteine by human erythrocytes. *The International Journal of Biochemistry and Cell Biology* 39(9): 1698-1706.

Raftos, J. E., T. M. Dwarte, et al. (2006). Direct measurement of the rate of glutathione synthesis in 1-chloro-2,4-dinitrobenzene treated human erythrocytes. *Redox Report* 11(1): 9-14.

Rice-Evans, C. and E. Baysal (1987). Iron-mediated oxidative stress in erythrocytes *The Biochemical Journal* 244: 191 - 196.

Richie, J. J., P. Abraham, et al. (1996). Long-term stability of blood glutathione and cysteine in humans. *Clinical Chemistry* 42(7): 1100-1105.

Richie, J. J., L. Skowronski, et al. (1996). Blood glutathione concentrations in a large-scale human study. *Clinical Chemistry* 42: 71-75.

Richman, P. and A. Meister (1975). Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. *Journal of Biological Chemistry* 250(4): 1422-1426.

Robertson, R. P., J. Harmon, et al. (2003). Glucose toxicity in b-cells: Type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes* 52: 581-587.

Rosen, E. D. and B. M. Spiegelman (2006). Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 444: 847-853.

Rossi, R., A. Milzani, et al. (2002). Blood glutathione disulfide: in vivo factor or in vitro artifact? *Endocrinology and Metabolism* 48(5): 742-753.

Sailaja, Y. R., R. Baskar, et al. (2003). The antioxidant status during maturation of reticulocytes to erythrocytes in type 2 diabetes. *Free Radical Biology and Medicine* 35(2): 133-139.

Sampathkumar, R., M. Balasubramanyam, et al. (2005). Increased glutathionylated hemoglobin (HbSSG) in type 2 diabetes subjects with microangiopathy. *Clinical Biochemistry* 38(10): 892-899.

Schafer, F. Q. and G. R. Buettner (2001). Redox environment of the cell as viewed through the REDOX state of the GSSG/GSH couple. *Free Radical Biology and Medicine* 30(11): 1191-1212.

Seghrouchni, I., J. Drai, et al. (2002). Oxidative stress parameters in type I, type II and insulin-treated type 2 diabetes mellitus; insulin treatment efficiency. *Clinica Chimica Acta* 321: 89-96.

Sies, H. (1985). *Oxidative stress: oxidants and antioxidants*. New York, Academic Press.

Silverthorn, D. U. (2007). *Human Physiology*. San Francisco, Pearson Benjamin Cummings.

Sinclair, A. J., A. J. Girling, et al. (1992). An investigation of the relationship between free radical activity and vitamin C metabolism in elderly diabetic subjects with retinopathy. *Gerontology* 38: 268-274.

Snoke, J. (1955). Isolation and properties of yeast glutathione synthetase. *The Journal of Biological Chemistry* 213: 813.

Snoke, J. and K. Bloch (1954). *Glutathione-A Symposium*. NY, Academic Press.

Snoke, J., S. Yanari, et al. (1953). Synthesis of glutathione from γ-glutamylcysteine. *the Journal of Biological Chemistry* 201: 573.

Sorensen, S., E. Rubin, et al. (1990). The role of membrane skeletal-associated α-globin in the pathophysiology of β-thalassemia. *Blood* 75: 1333-1336.

Srivastava, A. K., Y. Awasthi, et al. (1976). Studies on γ-glutamyl transpeptidase in human and rabbit erythrocytes. *Blood* 47(4): 645-650.

Srivastava, S. and E. Beutler (1969). The Transport of Oxidised Glutathione from human Erythrocytes. *Journal of Biological Chemistry* 244(1): 9-16.

Straface, E., R. Rivabene, et al. (2002). Structural changes of the erythrocyte as a marker of non-insulin-dependent diabetes: protective effects of N-acetylcysteine. *Biochemical and Biophysical Research Communications* 290: 1393-1398.

Sullivan, R. (1996). Contributions to senescence: non-enzymatic glycosylation of proteins. *Archives of Physiology and Biochemistry* 104(7): 797-806.

Tho, L., J. Candlish, et al. (1988). Correlates of diabetes markers with erythrocytic enzymes decomposing reactive oxygen species. *Annals of Clinical Biochemistry* 25: 426-431.

Thornalley, P. (1988). Modification of the glyoxalase system in human red blood cells by glucose *in vitro*. *Journal of Biochemistry* 254: 751-755.

Thornalley, P., A. McLellan, et al. (1996). Negative association between erythrocyte reduced glutathione concentration and diabetic complications. *Clinical Science* 91(5): 575-582.

Tietze, F. (1969). Enzymatic method for quantitative determination of nanogram amounts of total and oxidised glutathione applications to mammalian blood and other tissues. *Analytical Biochemistry* 27: 502-522.

Van Gaal, L. F., I. L. Mertens, et al. (2006). Mechanisms linking obesity with cardiovascular disease. *Nature* 444: 875-880.

Vijayalingam, S., A. Parthiban, et al. (1996). Abnormal antioxidant status in impaired glucose tolerance and non-insulin-dependent diabetes mellitus. *Diabetic Medicine* 13: 715-719.

Wautier, J. L. and A. M. Schmidt (2004). Protein glycation: a firm link to endothelial cell dysfunction. *Circulation Research* 95: 233-238.

Whillier, S., J. Raftos, et al. (2008). Glutathione synthesis by red blood cells in type 2 diabetes mellitus. *Redox Report* 13(6): 277-282.

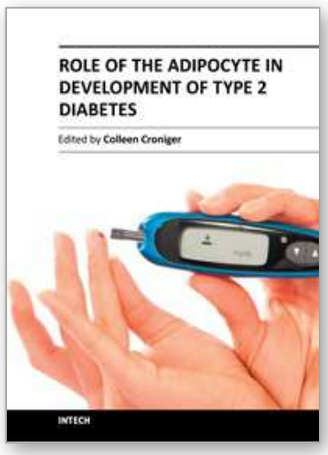
Wolff, S. P. and R. T. Dean (1987). Glucose autoxidation and protein modification. *The Biochemical Journal* 245: 243-250.

Wu, G., Y. Fang, et al. (2004). Glutathione metabolism and its implications for health. *Journal of Nutrition* 134(3): 489-492.

Yang, C., S. Chou, et al. (1995). Effect of ageing on human plasma glutathione concentrations as determined by high-performance liquid chromatography with fluorimetric detection. *Journal of Chromatography: Biomedical Sciences and Applications* 674(1): 23-30.

Yang, Y., M. Z. Dieter, et al. (2002). Initial characterization of the glutamate-cysteine ligase modifier subunit Gclm(-/-) knockout mouse - Novel model system for a severely compromised oxidative stress response. *Journal of Biological Chemistry* 277(51): 49446-49452.

Yoshida, K., J. Hirokawa, et al. (1995). Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. *Diabetologia* 38: 201-210.



Role of the Adipocyte in Development of Type 2 Diabetes

Edited by Dr. Colleen Croniger

ISBN 978-953-307-598-3
Hard cover, 372 pages
Publisher InTech
Published online 22, September, 2011
Published in print edition September, 2011

Adipocytes are important in the body for maintaining proper energy balance by storing excess energy as triglycerides. However, efforts of the last decade have identified several molecules that are secreted from adipocytes, such as leptin, which are involved in signaling between tissues and organs. These adipokines are important in overall regulation of energy metabolism and can regulate body composition as well as glucose homeostasis. Excess lipid storage in tissues other than adipose can result in development of diabetes and nonalcoholic fatty liver disease (NAFLD). In this book we review the role of adipocytes in development of insulin resistance, type 2 diabetes and NAFLD. Because type 2 diabetes has been suggested to be a disease of inflammation we included several chapters on the mechanism of inflammation modulating organ injury. Finally, we conclude with a review on exercise and nutrient regulation for the treatment of type 2 diabetes and its co-morbidities.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Stephney Whillier, Philip William Kuchel and Julia Elizabeth Raftos (2011). Oxidative Stress in Type II Diabetes Mellitus and the Role of the Endogenous Antioxidant Glutathione, Role of the Adipocyte in Development of Type 2 Diabetes, Dr. Colleen Croniger (Ed.), ISBN: 978-953-307-598-3, InTech, Available from: http://www.intechopen.com/books/role-of-the-adipocyte-in-development-of-type-2-diabetes/oxidative-stress-in-type-ii-diabetes-mellitus-and-the-role-of-the-endogenous-antioxidant-glutathione

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen