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COMMENTARY

Use of *In Vitro* Methaemoglobin Generation to Study Antioxidant Status in the Diabetic Erythrocyte*

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ABSTRACT. Poor glycaemic control in diabetes and a combination of oxidative, metabolic, and carbonyl stresses are thought to lead to widespread non-enzymatic glycation and eventually to diabetic complications. Diabetic tissues can suffer both restriction in their supply of reducing power and excessive demand for reducing power. This contributes to compromised antioxidant status, particularly in the essential glutathione maintenance system. To study and ultimately correct deficiencies in diabetic glutathione maintenance, an experimental model would be desirable, which would provide *in vitro* a rapid, convenient, and dynamic reflection of the performance of diabetic GSH antioxidant capacity compared with that of non-diabetics. Xenobiotic-mediated *in vitro* methaemoglobin formation in erythrocytes drawn from diabetic volunteers is significantly lower than that in erythrocytes of non-diabetics. Aromatic hydroxylamine-mediated methaemoglobin formation is GSH-dependent and is indicative of the ability of an erythrocyte to maintain GSH levels during rapid thiol consumption. Although nitrite forms methaemoglobin through a complex GSH-independent pathway, it also reveals deficiencies in diabetic detoxification and antioxidant performance compared with non-diabetics. Together with efficient glycaemic monitoring, future therapy of diabetes may include trials of different antiglycation agents and antioxidant combinations. Equalization *in vitro* of diabetic methaemoglobin generation with that of age/sex-matched non-diabetic subjects might provide an early indication of diabetic antioxidant status improvement in these studies. BIOCHEM PHARMACOL **60**;10:1409–1416, 2000. © 2000 Elsevier Science Inc.

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THE NON-DIABETIC ERYTHROCYTE

Human erythrocytes are subject to huge stresses during their life span, from mechanical shearing forces to oxygen-mediated damage. These cells thus have evolved a variety of mechanisms to preserve their structural integrity, primarily to maintain their haemoglobin in a reduced state capable of oxygen carriage. As they lack a nucleus, no new enzyme systems can be expressed in response to exceptional biochemical circumstances, so their existing systems must have the capacity to respond to a wide range of external and internal changes in environment over a long cellular life span. Over 90% of the weight of an erythrocyte is haemoglobin, which is a stable, tetrameric, hydrophobic ferriprotein that reversibly binds oxygen, provided that its iron is maintained in the ferrous (Fe^{2+}) state [1]. In the process of binding oxygen, oxyhaemoglobin becomes a superoxo-ferrihaem ($Fe^{3+}O_2^{\cdot-}$) complex [2], and when tissue release of oxygen occurs, the haem iron is restored to its ferrous state. However, low levels of oxygen departing from haemoglobin as superoxide ($O_2^{\cdot-}$) result in the oxidation of the haemoglobin to methaemoglobin (Fe^{3+}), which does not carry oxygen [3]. The net positive charge of methaemoglobin leads it to bind to hydroxyl groups at alkaline pH, or water molecules at acid pH [4]. The formation of small quantities of methaemoglobin through the autoxidation of haemoglobin during oxygen carriage occurs constantly in normal erythrocytes, and NADH-dependent cytochrome b_5 methaemoglobin reductase (NADH diaphorase) efficiently restores haemoglobin from methaemoglobin. This enzyme ensures that usually less than 1% of haemoglobin is oxidised at any one time in healthy individuals [5].

Haemoglobin is also a reactive molecule, capable of gaining or losing electrons relatively easily [1]. It can catalyse the oxidation of a variety of xenobiotics ranging from styrene [6] to aromatic amines [7], and methaemoglobin can result from these redox reactions. Superoxide, other oxygen radicals, hydrogen peroxide, and xenobiotic oxidants not only are capable of forming methaemoglobin, but also may cause attachment of haemoglobin to the cell cytoskeleton and expose senescence antigenic sites [8, 9]. As the appearance of these sites governs the splenic sequestration and destruction of senescent cells, oxidative damage alone is capable of severely curtailing the life span of the cell. In addition, reactive oxygen species may cause lipid peroxidation in the erythrocytic membrane, which

* Dedicated to the Memory of Michael Joseph Coleman, 1922–2000.

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reduces membrane fluidity [10] and impairs deformability of the cells [11]. In the long term, the clinical consequences of shortened erythrocytic life span and lack of deformability may include both acute and chronic effects, ranging from anaemia to microangiopathy.

EFFECTS OF GLYCATION IN DIABETES

It is now established that the majority of diabetic patients with poor glycaemic control suffer from increased risk of diabetic complications [12]. These are manifest in a variety of progressive disorders of the circulation, eyes, kidneys, and peripheral nervous system. To date, there is no unified theory that provides an acceptably detailed explanation as to how hyperglycaemia actually causes tissue damage and eventual organ failure. The Maillard AGE* hypothesis proposes that glucose levels that are consistently beyond the normal range (4–7 mM) lead to progressive non-enzymatic glycation. Briefly, the open chain aldehydic form of glucose facilitates its reaction with amino acids, structural proteins, and nucleic acids through a Schiff base condensation with amino groups [13]. After further rearrangement, the subsequent Amadori products undergo Maillard reactions, resulting in the highly reactive AGEs [14]. AGEs are believed to be cytotoxic [15], both cause and are formed by oxidative stress, and induce tissue damage [14]. However, the view that oxidative stress is the cause rather than the effect of many degenerative diseases has long been questioned [16], and a more recent perspective contends that oxidative stress alone is too narrow a source of AGE formation and that nonoxidative sources of reactive carbonyl species (carbonyl stresses) are also major contributors to the problem [17]. Thus, AGE-related tissue damage is a consequence of the failure of diabetic detoxification pathways to neutralise the reactive carbonyls [17]. Overall, a form of accelerated aging erodes the structure and therefore the function of virtually all proteins in diabetics.

In the erythrocyte, the effects on haemoglobin due to poor glucose control can result in more than 10% of haemoglobin existing as HbA_1. Decreased erythrocytic deformability is associated with high levels of HbA_1 [18], and erythrocytic AGEs induce oxidative stress in endothelial cells [19]. Diabetic tissues could be viewed as at risk from a triple threat: 'normal' reactive species production, AGE-related species formation, and, as increasing evidence suggests, deficits in antioxidant and detoxification enzymatic mechanisms.

* *Abbreviations*: AGE, advanced glycation end product; HbA_1, glycated haemoglobin; SOD, superoxide dismutase; GGCT, γ-glutamylcysteine transferase; HMPS, hexose monophosphate shunt; and G6PD, glucose-6-phosphate dehydrogenase.

ERYTHROCYTIC ANTIOXIDANT SYSTEMS IN DIABETES

SOD

A copper-zinc based enzyme, SOD is present in all cells as well as erythrocytes. It catalyses the conversion of two superoxide radicals to a molecule each of hydrogen peroxide and molecular oxygen [20]. Some authors have been unable to detect differences in SOD activity [21] between diabetic and non-diabetic subjects. Others have shown SOD glycation [22, 23], as well as reductions in red cell SOD activity in hyperglycaemia, consistent with glycation of the active site [24–26]. Overall, it is likely that most diabetic patients will suffer from a degree of impairment in this enzyme.

Catalase

Catalase converts hydrogen peroxide to water and molecular oxygen, and changes in this enzyme have been difficult to demonstrate in diabetes. Since the activities of both catalase and SOD are thought to be regulated by insulin [27], the hormone may also contribute to fluctuations of catalase activity in diabetes. Studies in leucocytes from diabetic patients and diabetic rats showed no changes in catalase activity [28–30]; however, in erythrocytes from diabetic patients, catalase levels were reduced in comparison with non-diabetic controls [26].

Glutathione Maintenance System

In both diabetic and non-diabetic tissues, the essential functions of GSH include co-factor or catalyst in many detoxification pathways, as well as the regulation of numerous proteins and enzymes [16, 17, 31]. However, its major role is that of direct and indirect maintenance of a reducing cellular environment. This is directly achieved by GSH-mediated neutralisation of reactive species, with and without the catalysis of GSH peroxidase. Indirectly, the glutathione maintenance system also provides the reducing power to maintain other tissue antioxidants in their reduced states, such as ascorbate and α-tocopherol [16]. In the erythrocyte, the major functions of GSH include protection from oxidant species generated by normal haemoglobin–oxygen interactions, as well as maintenance of haemoglobin and skeletal protein structure through continuous reduction of disulphide linkages. Commensurate with the exposure of the cell to oxidative risk, GSH erythrocytic levels are more than three orders of magnitude greater than those of the plasma [32, 33]. GSH levels are maintained by both synthesis and regeneration; synthesis of GSH from L-glutamate, glycine, and L-cysteine requires GGCT, which is rate-limiting, as well as GSH synthetase. GGCT is under negative feedback control from GSH itself [34]. During the process of GSH/GSH peroxidase-dependent reduction of toxic peroxides or superoxide, GSH is converted to GSSG, which is regenerated by GSSG reductase (also known as

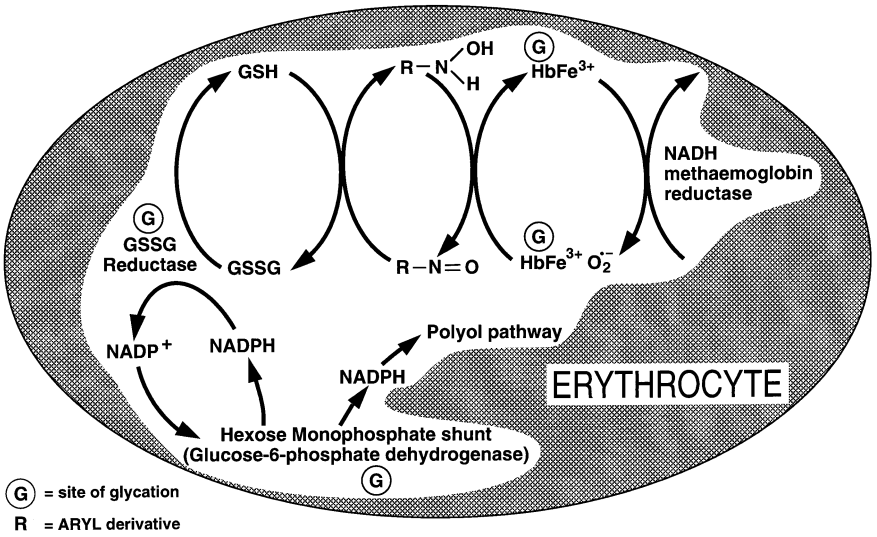

FIG. 1. Scheme representing aryl hydroxylamine-mediated methaemoglobin formation in the human erythrocyte in diabetes. The hydroxylamine oxidises a molecule of haemoglobin to methaemoglobin, while GSH reduces the resultant nitroso derivative to the hydroxylamine, which will in turn oxidise several other haemoglobin molecules in the same cyclic manner. The rapid phase of the process is sustained by GSH, which is maintained by GSSG reductase, an enzyme that requires NADPH from the HMPS. The major sites where non-enzymatic glycation may slow the process are illustrated, as well as the drain of reducing power through activation of the polyol pathway.

GSH reductase). Both synthesis and regeneration of GSH are energy-dependent processes, the former requiring ATP and the latter requiring NADPH supplied by the HMPS [31, 35]. The process is efficient enough to resynthesise the entire erythrocytic GSH complement in less than 10 min [36], and at any one time, 98% of the peptide is in the reduced form [37]. Indeed, GSH replenishment is so rapid that many experimental attempts to deplete the thiol have paradoxically caused an 'overshoot' increase in net GSH levels [33].

There has been some debate as to whether cellular glutathione levels are demonstrably lower in diabetics compared with non-diabetics, with some reports detecting decreases [26, 38, 39] while others show no clear differences [40, 41]. However, it is generally accepted that the glutathione maintenance system is impaired in diabetics [38, 42, 43]. Difficulties in identifying clear defects in GSH metabolism may have been accounted for partly by the considerable inter-individual variation generally seen within cellular defence systems, such as with GSH peroxidase [44]. Indeed, it is relatively difficult to demonstrate a clear deficiency in the ability of diabetic cells to resist experimental GSH depletion compared with non-diabetic erythrocytes [45]. It appears to be easier to reveal defects in GSH levels in newly diagnosed early stage type I patients, usually children and adolescents [39]. Diabetic erythrocytes show higher levels of GSSG egress compared with non-diabetic cells [46, 47], which could be a function of greater oxidative/carbonyl stress, as well as compromised maintenance of GSH levels. As the glutathione maintenance system is multifactorial, there are a number of levels where damage could occur due to glycation or AGEs. GSSG reductase has been reported to be less efficient in diabetics [46, 48], although this is not always the case with GSH peroxidase activity [42, 46]. However, G6PD, the main enzyme of the HMPS is believed to be compromised in diabetic subjects [36, 49]. In addition, erythrocytes respond to high glucose levels by activation of the polyol pathway, where increased activity of aldose reductase accentuates NADPH demand [50, 51]. Therefore, the deleterious combination of excess demand for reducing power as well as restriction in its supply is highly significant in explaining poor glutathione maintenance system performance in diabetes.

METHAEMOGLOBIN AS A MODEL OF CELLULAR STRESS

GSH-Amplified Methaemoglobin

Although direct measurement of GSH has not always illustrated deficiencies in the thiol maintenance system, a functional indication of glutathione maintenance system performance under stress would provide a more dynamically relevant illustration of any deficit in the ability of a cell to resist toxic insult. The process of aromatic amine-mediated methaemoglobin formation can provide this illustration, as it causes stress on glutathione maintenance in the erythrocyte. In the non-diabetic cell, hydroxylamines derived from aromatic amines react directly with the superoxo-ferrihaem complex ($Fe^{3+}O_2^{\cdot-}$) of oxyhaemoglobin, forming methaemoglobin (Fe^{3+}), hydrogen peroxide, and superoxide, as well as nitroso derivatives [52] (Fig. 1). Once formed, a nitroso derivative is readily reduced by the abundant cellular GSH to its hydroxylamine, which will oxidise more haemoglobin molecules through a co-oxidation cycle [32, 52–54]. In this way, one hydroxylamine molecule may oxidise up to four haemoglobin molecules in a rapid process

[32, 54]. Reduction of the hydroxylamine to its parent amine also occurs as a GSH-dependent byproduct of methaemoglobin formation [32]. Without a plentiful supply of GSH, haemoglobin oxidation cannot continue at this rapid rate and can also be attenuated by GSH depletors [55]. GSH-dependent hydroxylamine-mediated methaemoglobin formation frees GSSG reductase and the GSH synthetic system from their respective feedback controls, causing them to operate at a rapid rate. Consequently, the sudden demand for reducing power induces maximal activity from the HMPS [35]. In effect, the rapidity of methaemoglobin production is based on the extent of the recycling of the nitroso derivative, which is ultimately dependent on the efficiency of all the enzymes involved in erythrocytic GSH maintenance [56]. In a number of studies, diabetic erythrocytes have been demonstrated consistently to form less methaemoglobin than non-diabetic cells [55, 57, 58].

GSH-Amplified Methaemoglobin in Diabetic Erythrocytes

To determine the locus of low diabetic sensitivity to GSH-amplified methaemoglobin, initial studies compared the reactivity of haemoglobin between diabetics and non-diabetics. Although diabetic haemoglobin can be extensively glycated, there was no significant difference in methaemoglobin formed as a result of exposure of the hydroxylamine of monoacetyl dapsone (MADDS-NHOH) to purified haemoglobin or haemolysate in non-diabetics or diabetics [58]. This suggests that glycation itself does not change the reactivity of oxyhaemoglobin with the hydroxylamine. The difference between the cell types arises in studies with intact cells, suggesting that the problem lies in the glutathione maintenance system. Typically, during the first 3–5 min, hydroxylamine-mediated methaemoglobin formation is at its greatest velocity, and the process starts to plateau between 10 and 15 min. The deficiency in diabetic cellular methaemoglobin generation is clear in the first 3 min [55, 57, 58]. In the diabetic erythrocyte, it is unclear as to which stages in the methaemoglobin process are compromised due to glycation/AGE formation. Aside from possible deficiencies in GSSG reductase and GSH synthesis, the compromised reducing power supply from the HMPS is probably the critical factor in the response to methaemoglobin-mediated GSH consumption. The most severe example of poor HMPS function is hereditary G6PD deficiency, where oxidant xenobiotics have long been known to cause haemolysis [59]. The inadequate NADPH supply in G6PD-deficient patients [35] leads to lowered cellular GSH levels, which in turn causes them to form less methaemoglobin in response to aromatic amines such as dapsone than do normal individuals [59], although diabetics are not believed to be clinically G6PD-deficient [36]. Interestingly, high levels of GSSG can inhibit the activity of mammalian G6PD in conditions of low NADPH [60]. As increased flux of GSSG is seen through diabetic erythrocytes compared with non-diabetic cells [46, 47], it is possible that the low methaemoglobin formation in diabetic erythrocytes is linked with a GSSG-mediated restriction in the NADPH supply from HMPS to GSSG reductase.

In further investigations on methaemoglobin formation, it was found that the difference between non-diabetic and diabetic cells was lost after a 16-hr 37° incubation of both cell types at 10 and 20 mM glucose; diabetic methaemoglobin formation was shown to be restored to that of non-diabetics [58]. It is difficult to understand how glucose incubation alone could effect this change. In addition, at the glucose concentrations selected, it also seems unlikely that the reducing power drain through the polyol pathway would have been curtailed. It is possible that over the 16 hr of the study, the formation of glucose metabolites such as pyruvate may have affected the methaemoglobin-forming capability. A recent study *in vitro* showed that pyruvate and several antioxidants prevented the loss of activity of lens G6PD during incubation with increasing fructose concentrations [49]. However, as 'normal' methaemoglobin levels had been produced in the incubated diabetic erythrocytes, this indicates that reducing power supplies and the function of GSSG reductase and GSH synthesis must have been restored to non-diabetic levels by the conditions of the experiment. It is conceivable that the exposure to steady glucose concentrations may have reduced GSSG flux through the cells and diminished any GSSG-mediated restriction in the supply of NADPH from HMPS to GSSG reductase. This study does suggest that compromised function of key antioxidant enzyme systems in diabetic erythrocytes may not be irreversible [58], which outlines some potential for the chemical modulation of diabetic tissue detoxification defences.

Nitrite-Mediated Methaemoglobin Formation

The mechanism of nitrite-mediated methaemoglobin formation has not been fully elucidated to date, due to its complexity. The process has been described as occurring in two stages, a slow initial first-order single electron transfer to the bound dioxygen of oxyhaemoglobin, followed by a rapid autocatalytic amplification process, which was thought to be mediated by the superoxide formed in the slow phase [61] (Fig. 2). Later authors suggested that hydrogen peroxide formed during the initial slow stage of the process converts methaemoglobin to a free-radical intermediate, which mediates the autocatalytic stage [62, 63]. The most recent studies indicate that nitric oxide formed during the oxidation of haemoglobin by nitrite is crucial to the progress of the initial phase [64]. It has also been suggested that the accepted mechanism is not autocatalytic but relies on a reaction between oxyhaemoglobin and nitrogen dioxide [65].

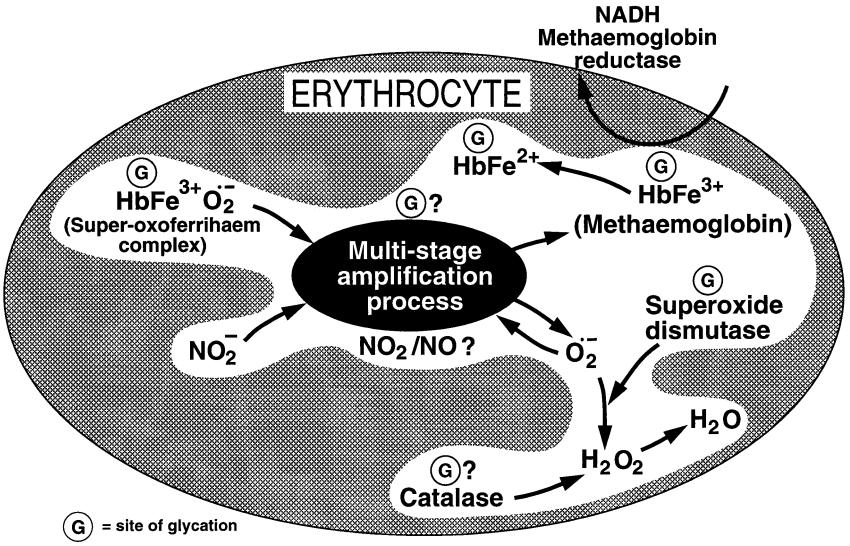

FIG. 2. Theoretical scheme representing nitrite-mediated methaemoglobin formation in the human erythrocyte in diabetes. Nitrite oxidises haemoglobin through a complex amplification process, where a number of nitrogen derivatives are thought to be involved. Where non-enzymatic glycation potentially could influence antioxidant function in the process is indicated.

Nitrite-Mediated Methaemoglobin in Diabetic Erythrocytes

Although the mechanism of nitrite-mediated methaemoglobin formation is independent of GSH and is markedly different from that of hydroxylamines, diabetic erythrocytes nevertheless form less nitrite-mediated methaemoglobin compared with non-diabetic cells [55]. In this case, it is difficult to determine which aspect of diabetic erythrocytic metabolism is compromised, as haemoglobin oxidation by nitrite is not completely understood. As with hydroxylamines, the diabetic intact cells were less susceptible to nitrite-induced methaemoglobin formation compared with non-diabetic cells, but there was no difference in the reactivity of the respective purified haemoglobins with nitrite [55]. It is likely that the difference in susceptibility to methaemoglobin generation, as with the glutathione-dependent toxins, lies in the cytosol of the erythrocytes rather than the haemoglobin itself.

Some agents actively inhibit nitrite-mediated methaemoglobin generation, such as urate, which retards the process by reacting with oxyhaemoglobin to form a hydroxyhaemoglobin radical [66]. It might be suggested that glucose-related products, such as those formed in the polyol pathway, might protect haemoglobin from oxidation in diabetics. However, in the *in vitro* studies that showed the differential with nitrite, the erythrocytes had been washed three times and were exposed to the same glucose concentration as the non-diabetic cells, 10 mM [55]; thus, it is probable that any abnormal byproducts of glucose metabolism in the diabetic cells would be removed by the washing process. Another possible explanation of the effects of nitrite in this study may be indirectly due to non-enzymatic glycation. The autocatalytic phase of nitrite-mediated methaemoglobin may depend on some form of redox-cycling of oxygen or nitrite-related species, which might conceivably require the activity of SOD or catalase. Indeed, catalase has been shown to reduce the velocity of nitrite-mediated methaemoglobinaemia [61] by preventing the peroxide-mediated formation of methaemoglobin radical [63]. It might be speculated that low diabetic erythrocytic susceptibility to nitrite-mediated methaemoglobin may be linked with differences in the functional capabilities of cytosolic antioxidant defence mechanisms other than GSH.

ROLE OF ANTIOXIDANTS IN DIABETES

It is clear that oxidative, carbonyl, and metabolic stresses in diabetes are present upon early onset of type I diabetes and indeed increase by early adulthood [39]. The consequences of early onset diabetes include ischaemic disease of the heart, retina, and intestine [11]. To protect diabetic tissues, much effort has been directed at modifying or reversing glycation by the use of agents such as aminoguanidine, which reached Phase III Clinical Trial, although the complete mechanisms of action of antiglycation agents are not known at present [67]. It is probable that even in the event of a successful antiglycation agent reaching the clinic, diet supplementation with antioxidants such as α-tocopherol, α-lipoic acid, and ascorbic acid [68, 69] will be recommended to provide protection for diabetic tissues. Indeed, vitamin E is already in use in some areas to protect diabetic children from microvascular complications [70]. However, antioxidant therapy has not been as successful as had been hoped in retarding the progress of diabetes and other diseases thought to be rooted in oxidative stress [17]. Theoretically, if reducing power is at a premium in diabetes, GSSG flux is abnormally high, and GSH maintenance is also inefficient, antioxidant supplementation should spare GSH from the burden of reducing other 'spent' tissue

antioxidants such as α-tocopheroxy radicals and dehydroascorbic acid. More GSH then should be available for non-oxidative detoxification pathways, which would in turn reduce the carbonyl stress seen in diabetes [17]. The lack of clear-cut clinical benefit of some antioxidant therapies may be due to a number of factors. Both exogenous and endogenous antioxidants can show pro-oxidant effects depending on their concentration and the specific oxidative process involved [17, 55, 71, 72]. As this effect is concentration/dose-related [72], more refinement in antioxidant dosage/benefit ratios may be necessary. It is also apparent that tissues require antioxidant capacity throughout their lipophilic as well as aqueous phases, necessitating the activity of molecules that differ widely physicochemically and structurally, but that carry out essentially the same function. It is hoped that future studies with diabetic patients will define precise combinations and dosages of antioxidants that would reproduce non-diabetic tissue antioxidant ratios accurately and minimize tissue aging.

METHAEMOGLOBIN MODEL: POSSIBLE APPLICATIONS IN DIABETES

Clinical Applications

The process of AGE formation and the evolution of tissue damage in diabetes may take place over many years. Presently, the most effective method of minimizing, but not removing, the risks of diabetic complications is control of glucose levels [17]. With the aim of the elimination of complication risk, future therapies might include anti-AGE agents, antioxidant supplement combinations, and tight monitoring of glucose concentrations. In any case, it will be essential to evaluate quickly the efficacy of any given therapeutic regimen aimed at the attenuation of complication development. One useful and rapid index of improved antioxidant capacity would be a sustained restoration of the ability of erythrocytes drawn from diabetics to form methaemoglobin levels in the range of cells from age and sex-matched non-diabetics. Samples drawn from patients in clinical trials of anti-complication therapy would be exposed *in vitro* to aromatic amine hydroxylamines and nitrites both before and during the commencement of treatment. The experimental assumption would be made that rapid improvement of methaemoglobin formation ability would indicate at least partial restoration of antioxidant capacity in the erythrocytes, and, one hopes, other tissues. This assumption remains to be proved experimentally, although it is not unreasonable to assume that if GSH performance were to be indistinguishable from that of non-diabetics, the cumulative risks of tissue damage in the subjects would be reduced. The erythrocyte itself is a key cell in the clinical progress of diabetic complications. Abnormal haemorrheological properties such as increased blood viscosity, haematocrit, and aggregation as well as lack of deformability all contribute to diabetic angiopathy [17, 73].

Experimental Applications

Experimentally, there are already many effective *in vitro* cell culture models that are aimed at modelling the effects of high glucose levels, glycation, and oxidative/carbonyl stress. The erythrocytic methaemoglobin generation model may provide additional information, such as the efficacy of novel antioxidant combinations *in vitro* in the presence of different methaemoglobin formers and diabetic/non-diabetic erythrocytes. From a practical viewpoint, erythrocytes drawn from diabetic patients are subject to the 'real-world' daily variation in glucose levels for a much longer period of time than shorter-lived experimentally maintained cells. They contain easily measured thiol levels and can be obtained and prepared quickly. In general, inter-individual variation is much less pronounced in erythrocytes compared with other cells such as mononuclear leucocytes and neutrophils [74–76]. Methaemoglobin formation is also rapid and is easily measured in the laboratory using a CO-Oximeter.

CONCLUSIONS

It is to be hoped that future strategies for the permanent restoration of endogenous insulin secretion are eventually successful, although their cost may restrict worldwide usage. It is probable that many diabetics will require biochemical protection from the lifetime consequences of a lack of precision in glucose concentration control. Usage of the *in vitro* methaemoglobin model to study the alleviation of the effects of oxidative stress could contribute to the evaluation of more effective antioxidant and antiglycation strategies.

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