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REDUCTION OF DAPSONE HYDROXYLAMINE TO DAPSONE DURING METHAEMOGLOBIN FORMATION IN HUMAN ERYTHROCYTES *IN VITRO* III: EFFECT OF DIABETES*

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Abstract—The fate of dapsone hydroxylamine has been investigated in diabetic and normal human erythrocytes. In erythrocytes from four type 1 (insulin dependent) diabetic subjects, there was a significant decrease in dapsone hydroxylamine-mediated methaemoglobin formation compared with cells drawn from normal individuals ($P < 0.01$). However, the ability of the diabetic cells to detoxify the hydroxylamine to dapsone was not correspondingly reduced and was not different to normal cells. The initial rate of the accelerating effect of diethyl dithiocarbamate (DDC) on hydroxylamine-mediated methaemoglobin and dapsone formation was significantly reduced in diabetic compared with normal cells. There was no significant difference in hydroxylamine-dependent methaemoglobin formation between diabetic erythrocytes pretreated with either statil or sorbinil and untreated diabetic cells. Dapsone recovery in diabetic erythrocytes incubated with statil was not significantly different from statil-free incubations. However, in the presence of sorbinil, there was a marked reduction in dapsone formation at all four time points, ($P < 0.001$ at 15 min). Mean measured levels of glutathione did not differ significantly between the normal (380 ± 30.9 mg/L; N = 8) and diabetic (349 ± 58.7 mg/L; N = 8) volunteers. In summary, although diabetic erythrocytes were less sensitive to the effect of dapsone hydroxylamine-mediated methaemoglobin formation in comparison with normal cells, glutathione-dependent hydroxylamine reduction to dapsone was unaffected.

Key words: metabolism; haemoglobin; oxidation; sulphone; sorbinil; statil

The anti-parasitic and anti-inflammatory properties of dapsone have led to an increasing number of therapeutic applications of this compound [1–5]. It is metabolised in man by cytochrome P450s to hydroxylamines [6], which are responsible for its haematological toxicity [7]. Within the erythrocyte, dapsone hydroxylamine-mediated methaemoglobin formation leads to the detoxification of the metabolite to the parent drug. Both of these processes are promoted by the relatively high glutathione levels found in erythrocytes compared with plasma [8, 9]. In type I diabetes, a variety of erythrocytic enzymes are functionally compromised, including glutathione reductase [10] and glucose 6-phosphate dehydrogenase [11]. Some studies have suggested this may lead to a depletion of glutathione within diabetic erythrocytes due to reduced availability of NADPH necessary for maintenance of glutathione levels [12]. Other studies have indicated glutathione levels to be similar to those of normal cells [11]. Dapsone hydroxylamine is known to exert oxidative stress on normal erythrocytes through glutathione depletion [8]. In addition, the shift in the oxygen dissociation curve in diabetic erythrocytes is believed to promote diabetic complications through hypoxia [13]. The effects of the hydroxylamine on diabetic erythrocytes have not so far been determined. In previous work, DDC§ has been used experimentally to study the relationship between dapsone hydroxylamine-mediated methaemoglobinaemia and subsequent dapsone formation in the human erythrocyte [7, 8]. In this work these processes have been investigated using dapsone hydroxylamine and DDC in diabetic as well as normal erythrocytes.

MATERIALS AND METHODS

Dapsone hydroxylamine was provided by the Jacobus Pharmaceutical Company Inc. (Princeton, NJ, U.S.A.), and was found to be 97% pure by HPLC. Dapsone and DDC were obtained from the Sigma Chemical Co. (Poole, U.K.). The internal standard for the HPLC assay (3,3′diaminodiphenyl sulphone) was obtained from the Aldrich Chemical Co. (Poole, U.K.). Inhibitors of aldose reductase, statil and sorbinil, were a gift from Dr Paul Thornally, University of Essex. All HPLC solvents were supplied by Fisons Ltd, (Loughborough, U.K.). Whole human blood was drawn from normal (mean age: 27.3 ± 4.0 years, N = 4) and diabetic volunteers (mean age: 26.7 ± 12.4 years; N = 4; glucose:

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§ Abbreviations: PBGS, phosphate-buffered (pH 7.4) 0.9% saline containing 10 mM glucose; DDC, diethyl dithiocarbamate.

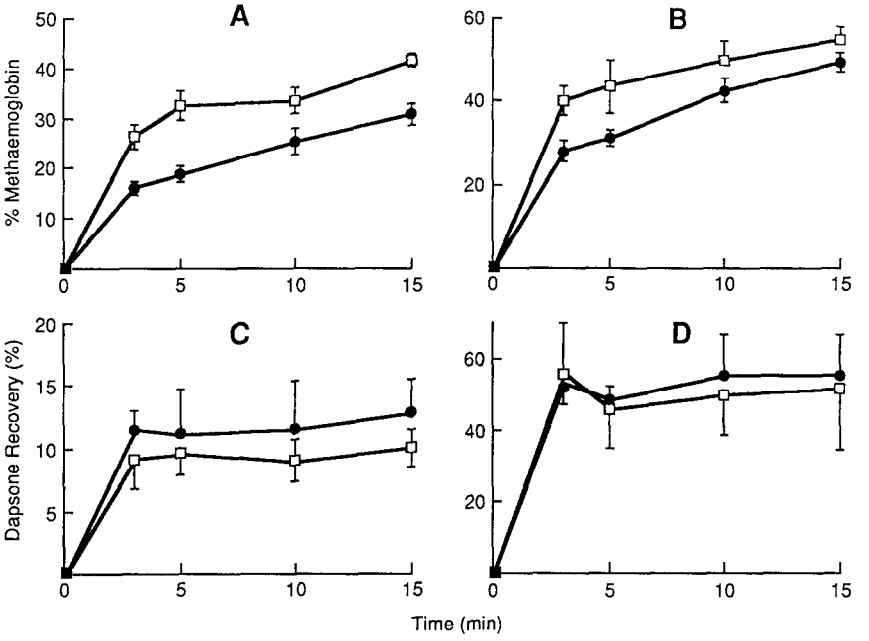

Fig. 1.(A) Dapsone hydroxylamine (DDS-NHOH; 150 μM)-dependent methaemoglobin formation; (B) DDS-NHOH-dependent methaemoglobin formation in the presence of DDC (5 mM). Diabetic (●); normal (□) erythrocytes (A and C scales differ); (C) dapsone recovery; (D) dapsone recovery in the presence of DDC (5 mM) after the administration of DDS-NHOH (150 μM). Diabetic (●); normal (□) erythrocytes. N = 12 per incubation, mean ± SD.

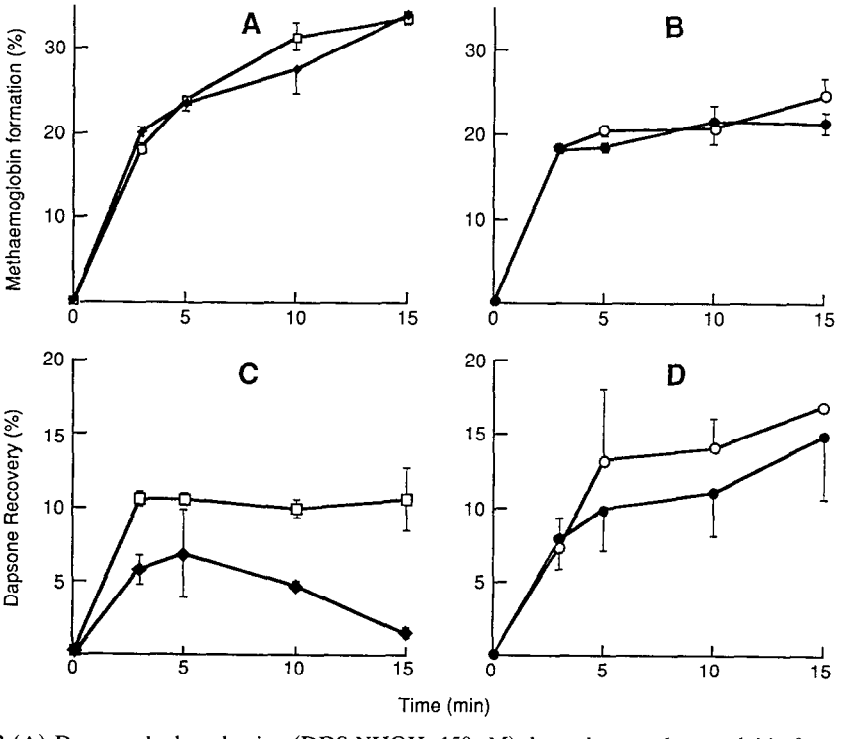

Fig. 2.(A) Dapsone hydroxylamine (DDS-NHOH; 150 μM)-dependent methaemoglobin formation in the presence (◆) and absence (□) of sorbinil. (B) DDS-NHOH (150 μM)-dependent methaemoglobin formation in the presence (●) and absence (○) of statil; (C) Dapsone recovery in the presence (◆) and absence (□) of sorbinil; (D) dapsone recovery in the presence (●) and absence (○) of statil in diabetic erythrocytes after the administration of DDS-NHOH (150 μM).

7.5 ± 6.4 mM), anti-coagulated with sodium heparin and placed on ice. The diabetic volunteers were all type 1 insulin dependent, otherwise healthy and were not taking any other medication. The erythrocytes were separated from the plasma and the buffy coat and top layer of cells removed. The cells were then washed twice in equal volumes of PBGS and resuspended to a 50% haematocrit also in PBGS. The erythrocyte incubations (0.5 mL) each contained 1.2 μmol of haemoglobin and were placed on ice to preserve intracellular glutathione concentrations and equilibrated for 10 min in uncapped tubes. All observations were carried out in triplicate per individual. Two main experimental groups were assembled: group A (data shown on Fig. 1) consisted of four sets. Set 1 contained erythrocytes from normal individuals; set 2 contained cells from diabetics; and sets 3 and 4 contained normal and diabetic cells, respectively, to which DDC (5 mM) had been added immediately prior to the addition of dapsone hydroxylamine. DDC is a potent accelerator of both erythrocytic processes of dapsone hydroxylamine-induced methaemoglobin formation and hydroxylamine detoxification [8, 9]. Group B (data shown on Fig. 2) also consisted of four sets: set 1 contained diabetic erythrocytes which had been pre-incubated with sorbinil (30 μM) for 30 min at 37° prior to being placed on ice. Set 2 contained diabetic erythrocytes which had been pre-incubated with buffer for 30 min at 37° prior to being placed on ice. Sets 3 and 4 were assembled in the same manner as sets 1 and 2 except the set 4 erythrocytes were pre-incubated with statil (30 μM). Both statil and sorbinil were added to the erythrocytes dissolved in methanol (5 μL). Dapsone hydroxylamine (0.075 μmol in 5 μL of acetone) was then added to the cooled erythrocytes (groups A and B) to give a final ratio of hydroxylamine to haemoglobin molecules of 1:16. Analysis of vehicle control incubations containing erythrocytes and either 5 μL of methanol or acetone revealed no effect on methaemoglobin formation compared with untreated erythrocytes. The samples were then gently mixed and placed in a 37° waterbath. The process of methaemoglobin formation due to compounds such as dapsone hydroxylamine and DDC is extremely rapid and temperature dependent [8, 9]. Therefore, to avoid inaccuracies due to these processes occurring in some samples before addition of the compounds was complete, the reactants were added to the red cells and thoroughly mixed while they were on ice and time zero was taken to be 15 seconds after they were placed in the waterbath to ensure a synchronous experiment. The effect of DDC alone on diabetic and normal erythrocytes was also determined under the experimental conditions stated above. Incubations were terminated at 3, 5, 10 and 15 min intervals. Aliquots (100 μL) were withdrawn for immediate methaemoglobin analysis using a IL-482 CO-oximeter (Instrumentation Laboratory, Warrington, U.K.) prior to the freezing of the samples to −20° until subsequent HPLC assay [14]. Erythrocytes from both normal and diabetic volunteers were assayed for glutathione levels employing a previously described method [15]. For glutathione assays, extra volunteers were recruited to bring the number of type I diabetics to N = 8 (mean age: 34.6 ± 15.2 years; glucose: 8.5 ± 5.4 mM), and normal volunteers to N = 9 (mean age: 28.3 ± 7.8 years). Recovery of dapsone from the compartments was measured by the HPLC assay in microgrammes, then converted to μmoles and expressed as a percentage of the number of μmoles of dapsone hydroxylamine originally added to the samples [9]. Statistical comparisons were made using Student's *t*-test accepting $P < 0.05$ as significant. Where more than one comparison was made with the same data, the acceptable level of significance was reduced to $0.05/k$ (where k is the number of tests) to compensate for the increased likelihood of reaching $P < 0.05$ during multiple testing [16].

RESULTS

In erythrocytes from four diabetic subjects, there was a significant decrease in dapsone hydroxylamine-mediated methaemoglobin formation compared with cells drawn from normal individuals (Fig. 1A < 0.001). In both diabetic and normal erythrocytes, there was a marked increase in methaemoglobin formation due to dapsone hydroxylamine in the presence of DDC (Fig. 1 A and B, $P < 0.001$). Methaemoglobin formation due to the hydroxylamine in the presence of DDC was significantly reduced in diabetic erythrocytes compared with normal cells at the 3 and 5 min time points ($P < 0.01$), but not at 10 and 15 min (Fig. 1B). Diabetic erythrocytes were also significantly less sensitive to methaemoglobinaemia induced by DDC alone compared with normal cells (Table 1).

The percentage of dapsone measured in the erythrocytes after the administration of dapsone hydroxylamine was not significantly different in normal compared with diabetic cells (Fig. 1C). In both diabetic and normal cells, there was a 4-fold increase in dapsone formation in the presence of DDC compared with hydroxylamine and erythrocytes alone (Fig. 1 C and D). However, in comparison with cells from normal donors, formation of dapsone from the hydroxylamine in the presence of DDC was not significantly different in diabetic cells (Fig. 1D), the ratio between methaemoglobin-dependent reduction of dapsone hydroxylamine to dapsone with and without DDC was not significantly different between diabetic (4.3 ± 1.17) and normal erythrocytes (4.19 ± 0.16) at 15 min. There was no significant difference in hydroxylamine-dependent methaemoglobin formation between diabetic erythrocytes pretreated with either statil or sorbinil and untreated diabetic cells (Fig. 2 A and B). Dapsone recovery in diabetic erythrocytes incubated with statil was not significantly different from statil-free incubations (Fig. 2D). However, in the presence of sorbinil, there was a marked reduction in dapsone formation at all four time points, ($P < 0.001$ at 15 min; Fig. 2C). Mean measured levels of glutathione did not differ significantly between the normal (380 ± 30.9 mg/L; N = 8) and diabetic (349 ± 58.7 mg/L; N = 8) volunteers (Fig. 3). However, two diabetic subjects exhibited markedly reduced glutathione levels, at 72 and 79% of the mean.

Table 1. Methaemoglobin formation due to DDC (5 mM) incubation with normal and diabetic erythrocytes (N = 4 per incubation, mean ± SD)

Time (min)	Methaemoglobin (%) Normal erythrocytes	Diabetic erythrocytes
3	5.7 ± 0.02*	4.8 ± 0.07*
5	6.3 ± 0.5†	4.9 ± 0.01†
10	6.8 ± 0.5‡	5.5 ± 0.3‡
15	7.9 ± 1.0§	6.0 ± 0.4§

* $P < 0.001$.
† $P < 0.01$.
‡ $P < 0.025$.
§ $P < 0.05$.

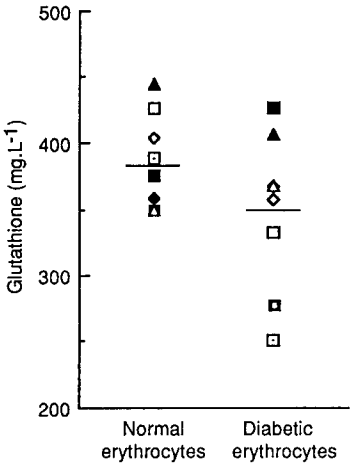

Fig. 3. Glutathione levels (mg/L) in normal (N = 9) and diabetic erythrocytes (N = 8).

DISCUSSION

Normal erythrocytes combat the effects of oxidative stress through the maintenance of a characteristically high intracellular GSH/GSSG ratio, which ensures the thiol-mediated protection of potentially reactive haemoglobin molecules from oxidation [11]. The intracellular levels of GSSG are low and unlike GSH it can pass through the cell's membrane [17]; indeed, erythrocytes release GSSG when under oxidative stress [17, 18]. GSH concentrations are normally maintained by the NADPH-dependent enzyme glutathione reductase [12]. Erythrocytic NADPH is supplied by the hexose monophosphate shunt, of which glucose 6-dehydrogenase is an essential component. Indeed, the cellular supply of glutathione is tightly controlled by allosteric negative feedback loops [19]. Erythrocytes in human diabetics have been shown in some studies to be unable to maintain normal GSH levels and demonstrate accelerated erythrocyte GSSG egress [20, 21]. This has been explained in terms of the reduced activities of glucose 6-phosphate dehydrogenase [11], glutathione reductase [10] and the increased activity of aldose reductase [12]. As aldose reductase is an NADPH-requiring enzyme its increased activity in response to hyperglycaemia [22] is believed to restrict the availability of NADPH for enzymes such as glutathione reductase. NADPH itself is also restricted in supply by the reduced function of the hexose monophosphate shunt. However, other studies have not demonstrated significant differences in GSH concentrations in diabetics compared with normal subjects [11, 23]. It is not clear whether diabetic erythrocyte GSH metabolism is significantly compromised.

When haemoglobin binds oxygen, iron is in a high spin state, having transferred an electron to the oxygen to form the superoxo–ferrihaem complex $Fe^{3+}O_2^-$ (Fig. 4) [24]. Dapsone hydroxylamine reacts with this complex to form methaemglobin (Fe^{3+}), a nitrosoarene and hydrogen peroxide [25]. Thus both haemoglobin and the hydroxylamine are oxidised. It is thought that the unstable nitrosoarene intermediate is then reduced by glutathione to the hydroxylamine which may then in turn oxidise another haemoglobin molecule. This futile cycling process may occur up to four times for each hydroxylamine molecule, before the hydroxylamine is either reduced to dapsone by intracellular thiols [8], the nitroso derivative covalently binds to the protein structure of the erythrocyte [26], or is oxidised to the nitro-derivative. Although aromatic amine hydroxylamine-mediated methaemoglobin formation will occur to a small extent in the absence of glutathione, the amplification and rapidity of the process is directly proportional to the availability of a comparatively large cellular pool of glutathione and other intracellular thiols. The redox cycling process is quickly curtailed when this pool is exhausted [8, 24, 25]. In normal human erythrocytes, the detoxification of dapsone hydroxylamine to dapsone is directly proportional to the rate of methaemoglobin formation and is curtailed once intracellular glutathione is depleted [8]. When DDC is incubated with erythrocytes treated with dapsone hydroxylamine it accelerates both methaemoglobin, dapsone formation and glutathione depletion [8]. Exactly how DDC amplifies these processes is unknown, although previous studies [24] have shown that DDC can itself bind to the superoxoferrihaem form of haemoglobin and oxidise haemoglobin

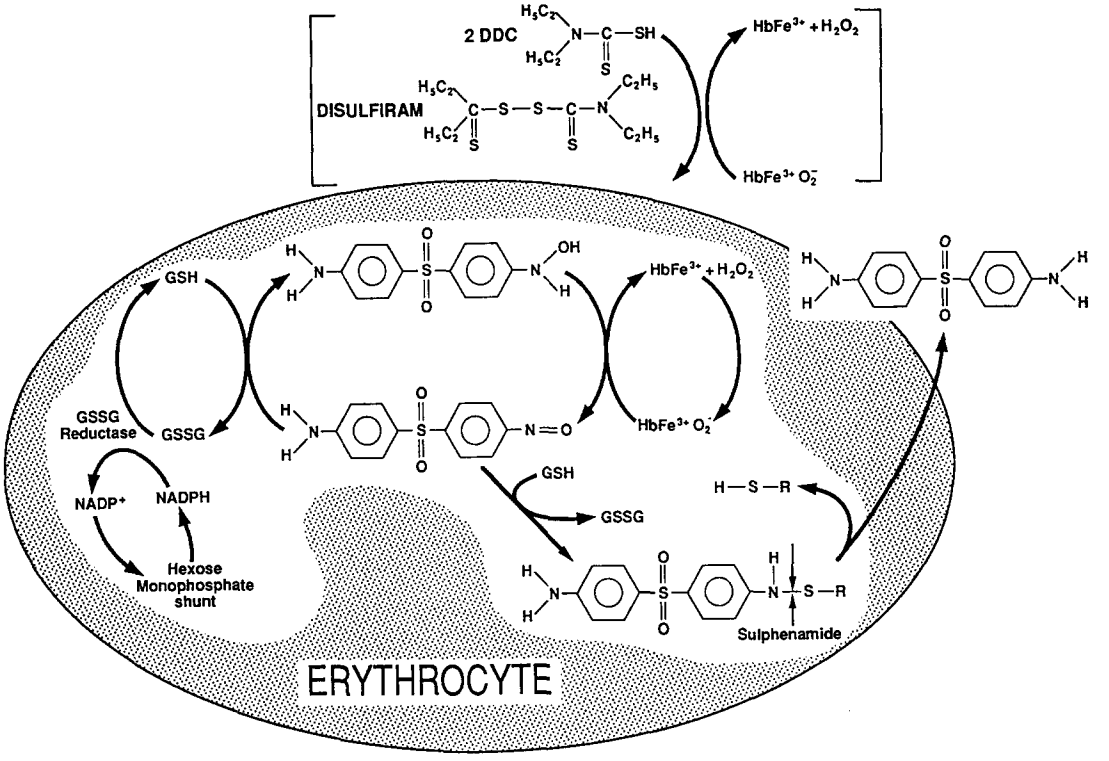

Fig. 4. Scheme of the co-oxidation of dapsone hydroxylamine, DDC and haemoglobin as well as dapsone formation within the erythrocyte.

through a similar co-oxidation cycle to that of dapsone (Fig. 4), although the DDC-mediated process is much slower.

In the present study, although erythrocytes from all four diabetic patients showed a reduction in dapsone hydroxylamine-mediated methaemoglobin formation compared with normal cells, this was not accompanied by a concomitant fall in hydroxylamine reduction to dapsone. Thus the relationship between the two processes of haemoglobin oxidation and dapsone detoxification is not linear in diabetic erythrocytes. Similarly, the increase caused by DDC on dapsone hydroxylamine-dependent methaemoglobin formation was lower in diabetic erythrocytes compared with normal cells. However, there was no difference in dapsone formation between normal and diabetic erythrocytes in the presence of DDC.

In this study, diabetic erythrocytes possessed sufficient glutathione to detoxify dapsone hydroxylamine as capably as normal cells. In addition, resting glutathione levels were not found to be significantly lower in diabetic erythrocytes compared with normal cells, which has been shown using larger numbers in previous studies [11, 23]. However, the accelerating effect of DDC, which is glutathione-dependent [7] was not as well accommodated at the early time points in diabetic cells compared with normal cells. Therefore it is possible that the lack of sensitivity of diabetic erythrocytes to dapsone-dependent as well as DDC-mediated methaemoglobin formation may lie in compromised glutathione metabolism as well as in the reaction between these compounds and the haem Fe^{2+}. It is possible that the relatively modest glycation seen in diabetics [27] may reduce the rate of the reaction between the hydroxylamine and haemoglobin, perhaps by steric hindrance.

The hydroxylamine is itself unstable and at physiological pH and temperature has a half-life in buffer of 37 min [28]. In the oxidant environment of both normal and diabetic erythrocytes, the hydroxylamine is likely to spontaneously oxidise extremely rapidly to the nitroso derivative which then reacts with glutathione which reduces it to dapsone. Both the processes of hydroxylamine reaction with haemoglobin and reaction with glutathione ultimately lead to dapsone formation. In diabetic erythrocytes, it is possible that the inability of glycated haemoglobin to react with the hydroxylamine sufficiently rapidly causes a greater proportion of metabolite to be reduced by glutathione, either by a direct reduction of hydroxylamine to dapsone, or after auto-oxidation to the nitroso which then reacts with glutathione. The net effect would be that methaemoglobin formation is not as rapid or complete as in normal cells, but reduction of the hydroxylamine is unaffected.

Dapsone hydroxylamine-dependent methaemoglobin generation is dependent on adequate glutathione supplies [7]. It was reasoned that inhibition of aldose reductase activity might promote glutathione availability by increasing the cellular

pool of NADPH and thus increase methaemoglobin generation. Incubation of diabetic erythrocytes with sufficient concentrations of statil and sorbinil to inhibit aldose reductase [29] did not affect dapsone hydroxylamine-dependent methaemoglobin generation in diabetic erythrocytes. Therefore it appears that aldose reductase activity has no appreciable effect on the ability of diabetic erythrocytes to sustain methaemoglobin formation. Statil did not affect hydroxylamine reduction to dapsone, although sorbinil caused a marked fall in dapsone formation through an unknown mechanism. It is conceivable that a combination of the degree of glycation related to control of glucose levels, genetic predisposition in rates of critical enzyme turnover and differences in glutathione metabolism may influence the toxicological interaction between xenobiotics and diabetic enzyme systems.

The oxygen release capacity of erythrocytes may be impaired in diabetics and studies in rats have indicated that diabetic neurological complications are linked to chronic hypoxia [30]. Therefore, in diabetics under treatment for dermatitis herpetiformis, the chronic methaemoglobinaemia which is a consequence of long-term use of high doses of dapsone necessary to control inflammatory disease may contribute to the development of diabetic complications. It may be that treatment regimens designed to reduce methaemoglobin formation [31] may be of some benefit in these patients.

In summary, erythrocytes from diabetic volunteers demonstrated less sensitivity compared with normal cells to dapsone hydroxylamine-mediated methaemoglobin formation although glutathione-mediated detoxification of the hydroxylamine was unaffected.

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