J. Pharm. Pharmacol. 1999, 51: 21–25
Received July 13, 1998
Accepted September 7, 1998

Resistance to Glutathione Depletion in Diabetic and Non-diabetic Human Erythrocytes In-vitro[a]

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Abstract

We have investigated the resistance of erythrocytes from diabetics and non-diabetics to glutathione depletion caused by *p*-benzoquinone, 1-chloro-2,4-dinitrobenzene (CDNB), diethyl maleate and 4-aminophenol.

Incubation of erythrocytes with 4-aminophenol (2 mM) caused a precipitous reduction (>80%) in cellular glutathione levels although there was no significant difference between 4-aminophenol-mediated glutathione depletion in the diabetic and non-diabetic cells. *p*-Benzoquinone and CDNB were both associated with a less severe initial reduction in glutathione levels (>50% at 30 min) although *p*-benzoquinone caused greater depletion ($P < 0{\cdot}001$) at 4·5 h (21·1 ± 3·1%, non-diabetic; 20·0 ± 1·0%, diabetic) compared with CDNB (49·2 ± 2·2%, non-diabetic; 51·3 ± 1·1% diabetic). Although there was no significant difference between the two types of cell in terms of level of depletion, administration of diethyl maleate caused a significant reduction in glutathione levels at 30 min ($P < 0{\cdot}0005$), 3·5 h ($P < 0{\cdot}05$) and 4·5 h ($P < 0{\cdot}05$) in erythrocytes from diabetic man compared with those from non-diabetic man. Co-administration of buthionine sulphoximine (20 mM) and 4-aminophenol (1 mM) also led to a significant reduction in glutathione levels in diabetic cells at 30 min ($P < 0{\cdot}05$), 3·5 h ($P < 0{\cdot}02$) and 4·5 h ($P < 0{\cdot}007$) compared with those in non-diabetic cells.

The observations that diabetic red cells' resistance to depletion was similar to that of non-diabetic cells for three of the four depletors, and that the combination of 4-aminophenol and buthionine sulphoximine-mediated inhibition of glutathione synthesis was required to illustrate differences suggests that diabetic complications might be a result of the long-term effect of small deficiencies in oxidative self-defence mechanisms such as glutathione.

Diabetes is associated with a variety of progressive disorders of the circulation, eyes, kidneys and peripheral nervous system. Recent studies have implicated a combination of oxidative stress and impaired antioxidant status as responsible for diabetic complications (Vijayalingam et al 1996). In diabetics high glucose concentrations lead both to formation of toxic reactive oxygen species (Guigliano et al 1995) and to a reduction in cell defence mechanisms such as glutathione reductase and superoxide dismutase through non-enzymatic glycation (Adachi et al 1991; Blakynty & Harding 1992). Hence, diabetics are less able to protect themselves and face a greater threat from reactive toxic species compared with non-diabetics. There is some debate about whether cellular glutathione levels are actually lower in diabetics than in non-diabetics — some workers have detected decreases (Yoshida et al 1995; Vijayalingam et al 1996) whereas others have not (Srivastava et al 1989; Di Simplicio et al 1995). However, it is likely that the glutathione maintenance system is impaired in diabetics (Yoshida et al 1995; Ohtsuka et al 1996).

In previous studies we used the glutathione-dependent process of xenobiotic-mediated methaemoglobin formation to illustrate differences between the glutathione maintenance systems of diabetics and those of non-diabetics (Coleman et al

[a]Dedicated to the memory of Dr M. J. Winn.

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1994, 1996, 1998). Although in a small study we were unable to demonstrate that erythrocyte glutathione levels were significantly lower in diabetics than in non-diabetics (Coleman et al 1994), we have shown consistently that methaemoglobin formation is greater in non-diabetics than in diabetics, suggesting that overall glutathione availability is higher in erythrocytes from non-diabetics than in those from diabetics.

In the current study, to investigate cellular glutathione availability more directly, erythrocytes both from non-diabetics and from diabetics were exposed to four different glutathione-depleting agents and to one depletor combined with buthionine sulphoximine, an inhibitor of glutathione synthesis.

Materials and Methods

Chemicals

The test compounds, 1-chloro-2,4-dinitrobenzene (CDNB), *p*-benzoquinone, 4-aminophenol, diethyl maleate, buthionine sulphoximine and dimethylsulphoxide were obtained from Sigma (Poole, UK). Reagents for the glutathione assay (hydrochloric acid (20 mM), glutathione, 5-sulphosalycylic acid and 5,5′-dithiobis 2-nitrobenzoic acid) were also obtained from Sigma.

Erythrocyte preparation

Whole blood was drawn from normal (mean age $37{\cdot}3 \pm 9{\cdot}0$ years, $n = 4$) and diabetic (mean age $44{\cdot}7 \pm 8{\cdot}4$ years; $n = 4$; glucose $8{\cdot}5 \pm 4{\cdot}4$ mM) volunteers, anti-coagulated with sodium heparin and placed on ice. The diabetic volunteers were all type 1 insulin-dependent and were 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 phosphate-buffered pH 7·4 saline containing 10 mM glucose (PBGS) and resuspended to a 50% haematocrit, also in PBGS.

Experimental design

The washed erythrocytes were decanted into 0·5-mL samples, each containing 1·2 μmol haemoglobin. The cells were incubated with either *p*-benzoquinone (1 mM), 4-aminophenol (1 mM and 2 mM), CDNB (1 mM) (all added in dimethylsulphoxide to give a final solvent concentration of 1%) and diethyl maleate (2 mM, added in methanol, again to give a final solvent concentration of 1%). Buthionine sulphoximine (final concentration 20 mM) was added to the erythrocytes dissolved in 0·1 mM NaOH (final incubation pH was 7·8). Each depletor was added to the erythrocytes at time zero and sampling was performed in triplicate per individual and commenced at 0·5 h, then at 1, 1·25, 1·5, 2·5, 3·5 and 4·5 h. In the experiment with buthionine sulphoximine and 4-aminophenol the sulphoximine was added, incubated for 30 min, then the 4-aminophenol was added and sampling commenced 30 min later. Because a pilot study showed that 2 mM 4-aminophenol in combination with buthionine sulphoximine caused 100% glutathione depletion in less than 3 h, 4-aminophenol was used with buthionine sulphoximine at 1 mM. Sulphoximine alone was also incubated with both diabetic and non-diabetic cells and the extent of depletion of glutathione measured as described above. At time zero and at all subsequent time-points washed blood (50 μL) was removed and assayed for glutathione using the method of Anderson (1985). Glutathione was expressed as a percentage of the initial control value measured in drug-free washed erythrocytes. Statistical comparisons were performed by one-factor analysis of variance; $P < 0{\cdot}05$ was accepted as indicative of significance.

Results

Pilot studies were performed to determine appropriate concentrations of the depletors which would cause a reduction greater than 30% in glutathione levels in the red cells. Buthionine sulphoximine alone elicited no significant depletion of glutathione over 6 h incubation. Incubation of erythrocytes with 2 mM 4-aminophenol caused a precipitous reduction (>80%) in cellular glutathione levels which was sustained for the duration of the experiment (Figure 1A). There was no significant difference between 4-aminophenol-mediated glutathione depletion in diabetic and non-diabetic cells (Figure 1A). *p*-Benzoquinone and CDNB (Figures 1B, C) both elicited a less severe initial reduction in glutathione levels (45–50% at 30 min) although benzoquinone caused a greater depletion ($P < 0{\cdot}0001$) at 4·5 h ($21{\cdot}1 \pm 3{\cdot}1$%, non-diabetic; $20{\cdot}0 \pm 1{\cdot}0$%, diabetic) compared with CDNB ($49{\cdot}2 + 2{\cdot}2$%, non-diabetic; $51{\cdot}3 + 1{\cdot}1$% diabetic). There was no significant difference between the levels of depletion in the two types of cell (Figures 1B, C).

However, administration of diethyl maleate (Figure 2A) caused a significant reduction in glutathione levels at 30 min ($P < 0{\cdot}0005$), 3·5 h ($P < 0{\cdot}05$) and 4·5 h ($P < 0{\cdot}05$) in diabetic ery-

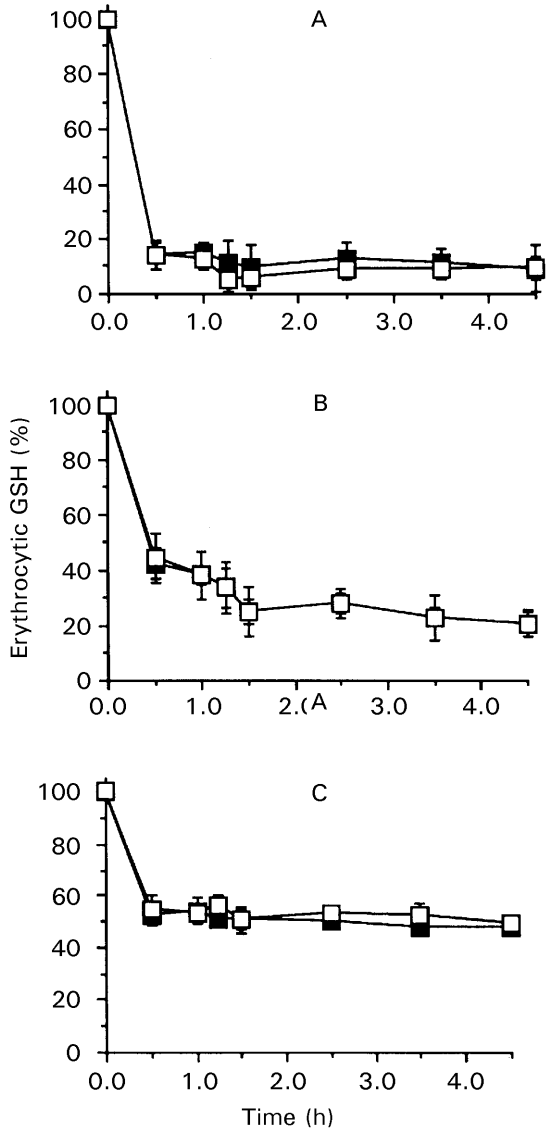

Figure 1. Percentage depletion of GSH in erythrocytes from diabetics (□) and non-diabetics (■) over 4·5 h after the pre-incubation of the cells with: A, 4-aminophenol (2 mM); B, *p*-benzoquinone; C, 1-chloro-2,4-dinitrobenzene. Results are means ± standard deviation; n = 9 per point.

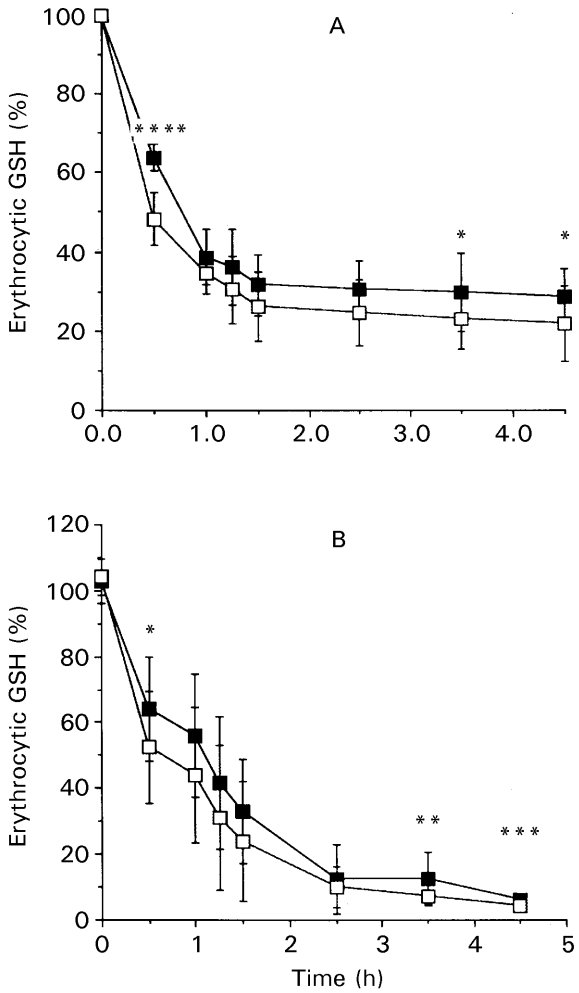

Figure 2. Percentage depletion of GSH in erythrocytes from diabetics (□) and non-diabetics (■) over 4·5 h after the pre-incubation of the cells with: A, diethyl maleate; B, buthionine sulphoximine (20 mM) and 4-aminophenol (1 mM). Results are means ± standard deviation; n = 12 per point. $*P<0{\cdot}05$, $**P<0{\cdot}02$, $***P<0{\cdot}007$, $****P<0{\cdot}0005$.

throcytes compared with those from non-diabetics. Co-administration of buthionine sulphoximine and 4-aminophenol (Figure 2B) also led to a significant reduction in glutathione levels in diabetic cells compared with those from non-diabetics at 30 min ($P<0{\cdot}05$), 3·5 h ($P<0{\cdot}02$) and 4·5 h ($P<0{\cdot}007$).

Discussion

The potent anti-oxidant protection provided by the glutathione system is of vital importance in the maintenance of tissue integrity throughout life. Glutathione levels fall in healthy individuals with increasing age (Yang et al 1995) and reduced levels of the thiol are associated with protein oxidation, especially in cataract formation (Babizhayev 1996). The system for maintenance of glutathione involves its synthesis (γ-glutamylcysteine synthetase, GGCS) and GSH synthetase, reduction from GSSG to glutathione (glutathione reductase) and glutathione peroxidase, which can catalyse the reaction of the thiol with peroxides (Seelig & Meister 1985).

Glutathione production is tightly regulated in erythrocytes — GGCS and glutathione reductase are inhibited by high levels of glutathione (Seelig & Meister 1985). In diabetics non-enzymatic glycation reduces the efficiency of glutathione reductase (Blakynty & Harding 1992). Difficulties in showing consistently that the diabetic system is compromised might in part be because of inter-individual variation of cellular defence systems, such as with glutathione peroxidase (Bolzan et al 1997). In addition, diabetic complications take

years to develop, indicating that differences in antioxidant capability are relatively modest but become highly significant over time.

In this study, there were no significant differences between the effects of the potent glutathione depletors CDNB, *p*-benzoquinone and 4-aminophenol in diabetics and non-diabetics despite their different modes of action. Benzoquinone derivatives deplete thiols by conjugate formation and by redox reactions (Albano et al 1985), whereas 4-aminophenol forms adducts with erythrocytic glutathione (Eckert 1988) and undergoes thiol-consuming methaemoglobin formation (Coleman et al 1998). CDNB reacts directly with glutathione and with glutathione transferase (Adams & Sikakana 1992). The use of such agents mimics an aspect of oxidative stress — glutathione is depleted but the process whereby more of the thiol is synthesized and GSSG is regenerated by glutathione reductase is intact. In view of the known deficiencies in diabetic glutathione maintenance, it is surprising that this system was able to withstand the depletion indistinguishably from that of non-diabetic erythrocytes, especially with potent agents such as *p*-benzoquinone and 4-aminophenol.

In contrast, diethyl maleate induced significant early and later differences between the resistance of diabetic and non-diabetic cells to glutathione depletion. Although diethyl maleate depletes the thiol in a similar manner to CDNB, it is converted to maleate which might interact with other erythrocytic enzymes — it reacts with many other thiols and is cytotoxic (Coleman et al 1994). This might at least partially account for the capacity of diethyl maleate to differentiate between diabetic and non-diabetic cellular resistance to glutathione depletion, whereas the other depleting agents could not. Additionally, a difference between the thiol-depleting action of CDNB and diethyl maleate has also been shown in platelets exposed to menadione (Cho et al 1996).

Direct depletors of glutathione should cause some rebound in synthesis of the thiol as the negative feedback inhibition is removed and synthesis is unencumbered. The combination of buthionine sulphoximine and 4-aminophenol caused almost complete glutathione depletion towards the end of the study period because the synthesis of the thiol was inhibited by the sulphoximine and the remaining pool of glutathione was exhausted. This combination caused differences between the extents of depletion in diabetic and non-diabetic cells, whereas either compound alone did not. As synthesis was blocked by buthionine sulphoximine, only glutathione reductase could be available for release from its negative feedback inhibition to convert GSSG to glutathione.

Glutathione reductase is less efficient in diabetics (Blakynty & Harding 1992) and it requires NADPH which is supplied mainly from the hexose monophosphate shunt. The main enzyme of the hexose monophosphate shunt is glucose-6-phosphate dehydrogenase, which is also impaired in diabetics (Costagliola 1990). In addition, polyol pathway activation restricts NADPH availability and is associated with reduced glutathione levels in diabetics (Bravi et al 1997). Reduced NADPH availability would further restrict the effectiveness of glutathione-reductase. Hence it is plausible that all these factors contribute to the differences between the resistance to depletion of diabetic and non-diabetic glutathione erythrocytic pools.

In a previous study in which methaemoglobin formation was used to model oxidative stress, the extent of the difference between diabetics and non-diabetics was strongly influenced by glucose concentration and duration of incubation (Coleman et al 1996). In this study, differences between resistance to glutathione depletion in diabetic and non-diabetic cells occurred both acutely within 30 min and chronically over some hours. This study involved small numbers and the differences between diabetic and non-diabetic cells were not easily shown using glutathione depletors. Overall, this complex picture of low-level impairment of diabetic resistance to oxidative stress could be viewed as commensurate with the gradual nature of development of diabetic complications.

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