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The role of aldose reductase inhibition in diabetic neutrophil phagocytosis and killing

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SUMMARY

This study examines whether an aldose reductase inhibitor (statil, ICI) can enhance neutrophil oxidative killing by diabetic neutrophils. We have examined a radiometric assay of phagocytosis and killing of *Candida albicans* by neutrophils from 20 controls and 20 subjects with insulin-dependent diabetes under various *in vitro* glucose concentrations. Glucose was present at 5, 10 and 20 mM in the presence and absence of statil (11 μM). Phagocytosis was unaffected by raised glucose levels in controls and in diabetic subjects. Killing by the diabetic cells was inhibited by increasing concentrations of glucose, killing was $18{\cdot}9 \pm 2{\cdot}0$, $16{\cdot}9 \pm 2{\cdot}4$ and $14{\cdot}8 \pm 2{\cdot}0\%$ (mean ± s.e.m.) at 5, 10 and 20 mM glucose, respectively ($P < 0{\cdot}05$). With the addition of statil under the same conditions killing improved to $19{\cdot}3 \pm 2{\cdot}0$, $23{\cdot}2 \pm 2{\cdot}2$ and $23{\cdot}6 \pm 2{\cdot}4$ ($P < 0{\cdot}01$), these values were similar to the controls ($P > 0{\cdot}01$). We conclude therefore that aldose reductase inhibition restores oxidative killing to normal.

Keywords diabetes neutrophils oxidative killing aldose reductase

INTRODUCTION

Patients with well controlled diabetes are no more prone to common infection than non-diabetic controls (Gocke, 1980), but a minority of poorly controlled patients develop severe and persistent infections (Reeves & Wilson, 1991). These infections are often difficult to eradicate and it is clear that they complicate the control of blood sugar, the requirement of insulin and can lead to ketoacidosis (Gocke, 1980). Hill *et al.* (1989) studied 51 diabetic outpatients and reported that a high glycosylated haemoglobin was an important risk factor in developing yeast infections. Similarly, correlations have been shown between mean plasma glucose levels and the frequency of acute bacterial infections (Rayfield *et al.*, 1982).

The neutrophil is the first haematological line of defence against infection (Nolan, Beaty & Bagdade, 1978), and clearly its function is relevant to the severity of infection once an organism is established.

Defects in neutrophil function such as chemotaxis (Bagdade, Root & Dulgar, 1974), phagocytosis (Geisler *et al.*, 1984; Gin, Boitter & Aubertin, 1984) and bactericidal killing (Tan *et al.*, 1975; Kaneshige *et al.*, 1982) have been reported in diabetes and have been related to hyperglycaemia.

In previous studies we have examined whether the metabolic disturbances of diabetes influences the metabolism within the neutrophil and its subsequent ability to ingest and kill an organism. We have shown that increased concentrations of glucose and 3-hydroxybutyrate inhibit the early oxidative-phase killing of *Candida albicans* (Wilson & Reeves, 1986) and superoxide production (Wilson, Tomlinson & Reeves, 1987) in diabetic neutrophils.

Oxidative killing occurs at an earlier stage than non-oxidative killing (Nathan, 1982). The initial step of the oxidative burst involves the conversion of molecular oxygen to superoxide by an NADPH-dependent membrane oxidase (Babior, 1978). The pentose phosphate pathway (PPP) produces the NADPH required for this process (Oren *et al.*, 1963). Superoxide is the first product produced in the respiratory burst and is the source of toxic oxygen products involved in oxidative killing.

Because excess glucose is able to reduce oxidative killing and superoxide anion production, we suggest that under hyperglycaemic conditions, there is competition for NADPH. The available NADPH is being channelled into another pathway, other than superoxide production.

One possible route is the polyol pathway, which at high glucose concentrations converts glucose to sorbitol using an NADPH-dependent enzyme aldose reductase.

The neutrophil in common with other tissues prone to diabetic complications has no requirement for insulin for glucose transport across the cell membrane (Wilson, 1986). It has glucose concentrations in equilibrium with plasma concentrations. Free glucose is not present within the neutrophil. Under conditions of hyperglycaemia, when glucose concentra-

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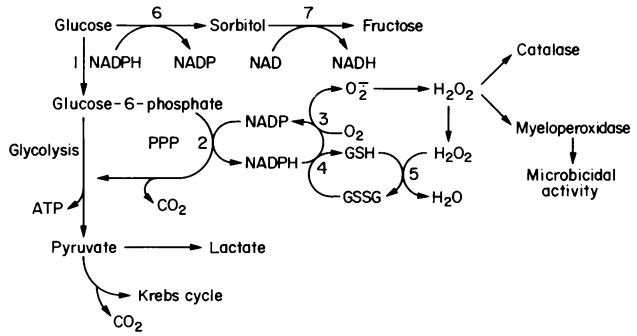

Fig. 1. Glucose metabolism in the neutrophil. 1, Hexokinase; 2, glucose-6-phosphate dehydrogenase and 6-phosphoglucoate dehydrogenase; 3, NADPH oxidase; 4, glutathione reductase; 5, glutathione peroxidase; 6, aldose reductase; 7, sorbitol dehydrogenase.

tions are in excess of that which can be phosphorylated to glucose-6-phosphate by hexokinase, glucose may be metabolized by aldose reductase (Fig. 1).

The affinity of aldose reductase for glucose is low (K_m 100–250 mM; Bedford *et al.*, 1988); therefore its activity becomes significant at elevated glucose concentrations.

Here we have used a radiometric assay to examine the *in vitro* effect of glucose and an aldose reductase inhibitor (statil; ICI, Alderley Edge, UK) on phagocytosis and killing of candida in patients with insulin-dependent diabetes and controls.

SUBJECTS AND METHODS

Subjects

Twenty patients (12 men, eight women; mean age 53 years, range 23–79) with insulin-dependent diabetes were studied. Their glycated haemoglobin was 8·99 ± 3·1% (mean ± s.d. Normal range 3·5–6·8%) and blood glucose 9·9 ± 3·8 mM (mean ± s.d. Normal range 3·5–5 mM).

In addition, 20 non-diabetic healthy subjects (mean age 52 years, range 19–76) were studied.

Preparation of neutrophils (PMN)

PMN were prepared from 30 ml of fresh heparinized whole blood by a method modified from Ferrante & Thong (1980, 1982). Briefly, 20 ml Hypaque was mixed with 90 ml Ficoll to produce a solution of specific gravity 1·114. Six millilitres of whole blood were layered onto 4 ml of this mixture in a conical centrifuge tube and spun at 500 ***g*** for 50 min. Mononuclear cells and platelets are held at the plasma: Ficoll–Hypaque interface, whereas PMN separate as a single band within the Ficoll–Hypaque layer and the red cells sediment to the bottom of the tube. PMN were washed three times in medium 199 (Flow Laboratories) and made up to 5×10^6/ml in medium. Cells were stained with gentian violet (0·04% in 3% acetic acid). Differential counting indicated a purity of about 98% PMN.

Preparation of C. albicans

Cultures of *C. albicans* were grown in Sabouraud dextrose medium (Oxoid, Basingstoke, UK) by inoculation from a Sabouraud agar slope culture and incubated for 24 h at 37°C. Organisms were washed three times in medium 199 before each assay. The candida were suspended at a concentration of 1×10^7/ml in medium 199.

Assay procedure

The assay depends on the fact that extracelluar viable candida incorporate ^{3}H-uridine whereas those killed do not. The procedure was as follows: assay tubes were set up in triplicate. Each tube contained 1×10^7 *Candida albicans* and 5×10^6 PMN suspended in 500 μl medium 199, containing 5% normal human serum. The first triplicate was prepared in medium 199 containing 5·6 mM glucose, each subsequent set of tubes had glucose added to achieve final concentrations of 10 and 20 mM glucose. Identical sets of experiments were carried out with the addition of an aldose reductase inhibitor, statil (ICI). Statil was added to test and control tube at a final concentration of 11 μM. The tubes were shaken in a waterbath at 37°C for 10 or 30 min.

Phagocytosis was assessed by taking a 100-μl aliquot from each tube into a microtitre plate containing 0·2 μCi ^{3}H-uridine.

Killing was measured by taking a 100-μl aliquot into a microtitre plate containing 0·2 μCi ^{3}H-uridine plus 0·6% sodium deoxycholate and DNAse (100 μg/ml) in 100 μl medium and incubating at 37°C for 60 min. After incubation the cells and candida were harvested onto filter discs using a Skatron cell harvester. The filters were dried, placed in scintillation fluid and the beta-emission was measured using a Beckman LS 5000 scintillation counter. The mean counts for the triplicate tubes were recorded and the phagocytosis and killing of *C. albicans* was expressed as percentage inhibition of ^{3}H-uridine uptake by the formula:

$$\left(1 - \frac{\text{ct/min with PMN}}{\text{ct/min without PMN}}\right) \times \frac{100}{1}$$

For phagocytosis, the percentage of inhibition was calculated from the counts obtained from the wells containing intact PMN.

Killing was calculated from the counts from wells containing deoxycholate and DNAse which disrupts the cells and releases the phagocytosed candida.

Osmolarity measurements

The osmolarity of the samples was measured on an advanced wide-range osmometer 3W II.

Statistical analysis

Student's *t*-test was used to compare baseline levels of phagocytosis and killing in diabetic and control neutrophils. The effect of increased glucose concentrations and the aldose reductase inhibitor within a group were analysed using analysis of variance. All calculations were performed on an RM Nimbus PC-286 using software from the Statistical Graphics Corporation.

RESULTS

Phagocytosis

Phagocytosis was measured after 10 and 30 min. The 10-min incubations were used to examine the early attachment and ingestion of candida and 30-min values the end-point of phagocytosis (Wilson & Reeves, 1986).

Baseline data at 5 mM glucose

There was no significant difference in the levels of phagocytosis between the controls and diabetic patients at 10-min and 30-min incubations ($P > 0·1$) (Tables 1 and 2).

Table 1. Examination of the effect of glucose and an aldose reductase inhibitor on phagocytosis and killing of *Candida albicans* (10-min levels)

	Controls ($n=20$) Glucose (mM)			Controls ($n=20$) Glucose (mM) with statil			Diabetics ($n=20$) Glucose (mM)			Diabetics ($n=20$) Glucose (mM) with statil		
	5	10	20	5	10	20	5	10	20	5	10	20
Phagocytosis (%)	70·6±1·4	70·4±1·4	71·4±1·2	72·5±1·4	71·8±1·3	71·9±1·2	68·9±2·2	72·0±2·1	71·2±1·7	72·6±1·7	73·6±2·0	71·9±1·7
Killing (%)	21·3±2·1	20·8±2·1	21·9±1·8	21·9±2·3	22·5±2·0	21·3±2·3	18·9±2·0	16·9±2·4	14·8±2·0*	19·3±2·0	23·2±2·2†	23·6±2·4†

* $P<0.05$ significant difference compared with 5 mM glucose.
† $P<0.01$ significant difference compared with percentage of killing at same glucose concentration without statil.

Table 2. Examination of the effect of glucose and an aldose reductase inhibitor on phagocytosis and killing of *Candida albicans* (30-min levels)

	Controls ($n=20$) Glucose (mM)			Controls ($n=20$) Glucose (mM) with statil			Diabetics ($n=20$) Glucose (mM)			Diabetics ($n=20$) Glucose (mM) with statil		
	5	10	20	5	10	20	5	10	20	5	10	20
Phagocytosis (%)	83·1±1·1	81·4±1·4	83·7±1·4	82·3±1·6	82·4±1·2	82·4±1·6	82·3±1·7	81·9±2·1	82·7±1·6	83·1±1·8	83·3±1·8	81·6±1·9
Killing (%)	64·7±1·2	64·2±1·4	64·4±2·0	64·2±2·0	64·9±1·6	63·6±1·7	62·1±2·6	61·0±2·9	61·8±2·9	61·8±2·9	62·8±2·9	61·2±2·8

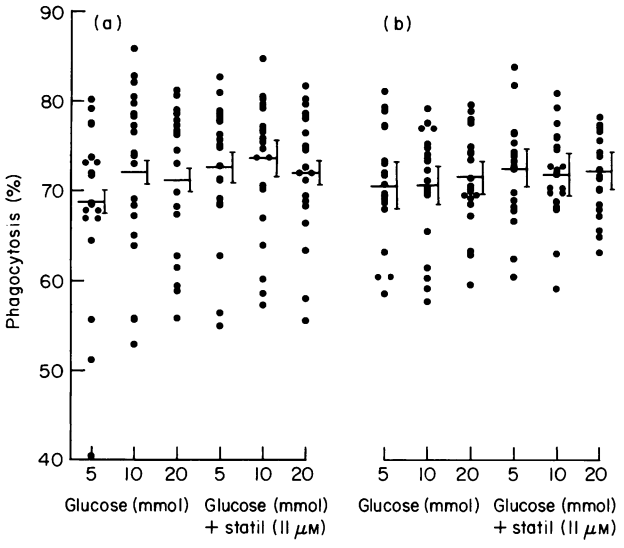

Fig. 2. Phagocytosis (%) of *C. albicans* by diabetic (a) and control (b) neutrophils under elevated glucose concentrations and in the presence of statil (11 μM); 10-min levels. Horizontal lines represent means ± s.e.m.

Effect of increased concentrations of glucose
No inhibition of phagocytosis was observed at either 10 mM or 20 mM glucose in either control of diabetic subjects ($P>0.1$) (Fig. 2).

Effect of aldose reductase inhibitor (statil)
No additional effect was seen with the addition of statil in the diabetic or control subjects (Tables 1 and 2).

10-minute killing incubations
Ten-minute incubations represented oxidative killing of candida by the neutrophils (Klebanoff & Clark, 1978).

Baseline data at 5 mM glucose: candida killing after a 10-min incubation was 21·3±2·1% (mean±s.e.m.) in the controls and 18·9±1·4% in the diabetic subjects. These values were not significantly different ($P>0.1$).

Effect of increased concentrations of glucose: there was no inhibition of candida killing with increased concentrations of glucose in the control subjects (Table 1, Fig. 3). The ability of the PMN from diabetic patients to kill candida was inhibited by increased concentrations of glucose. Mean killing values were 18·9±1·4, 16·89±2·4 and 14·8±2·1% at 5, 10 and 20 mM glucose, respectively (Fig. 4). The degree of inhibition at 20 *versus* 5 mM was significantly different ($P<0.05$).

Effect of aldose reductase inhibitor (statil). Statil had no significant effect on the killing ability of the control PMN (Table 1). With the addition of statil to the diabetic PMN killing improved to 19·3±2·0, 23·2±2·2 and 23·6±2·4% at 5, 10 and 20 mM glucose, respectively ($P<0.01$). These values were similar to those of controls ($P>0.1$).

Thirty-minute killing incubations
Killing of candida measured after a 30-min incubation period was normal in the controls and patients. This incubation period represented non-oxidative killing (Wilson, 1984). Statil had no additional affect on these killing values (Table 2).

Osmotic effect
The osmolarity in the experiments using 5 mM glucose was 421 mosmol/kg H_2O; this did not rise significantly when glucose

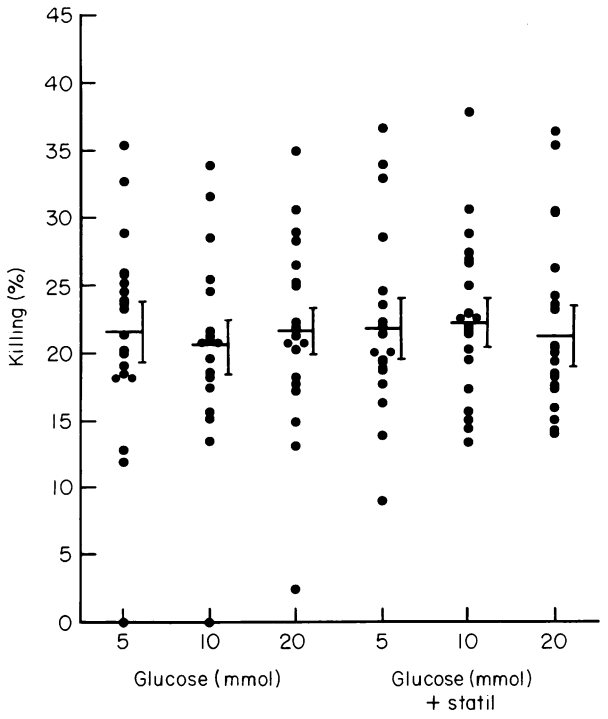

Fig. 3. Killing (%) of *C. albicans* by control neutrophils (10-min values). The horizontal bars represent means ± s.e.m.

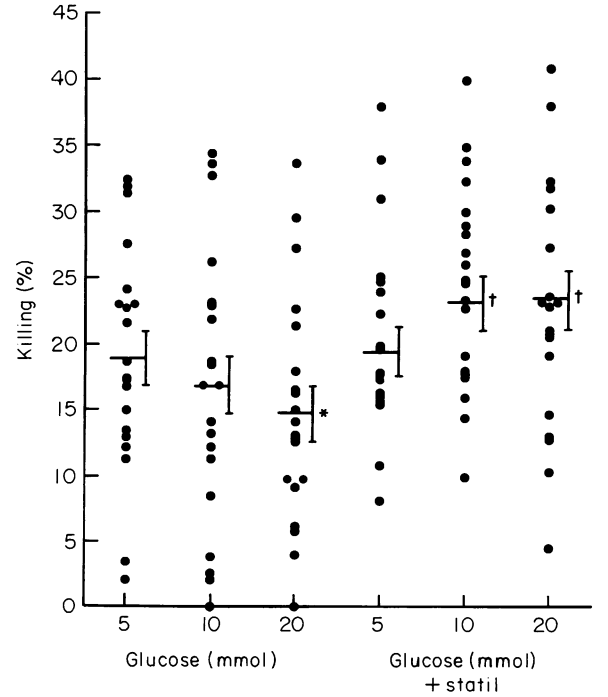

Fig. 4. Killing (%) of *Candida albicans* by diabetic neutrophils (10-min levels). The horizontal bars indicate means ± s.e.m. *$P > 0{\cdot}05$ compared with 5 mM glucose. †$P > 0{\cdot}01$ compared with values in the absence of statil.

concentrations were elevated to 10 and 20 mM. Values were 425 and 431 mosmol/kg H_2O, respectively. These results were not altered by the addition of statil (11 μM), values were 422, 425 and 432 for 5, 10 and 20 mM glucose, respectively.

From these results, any adverse effects on neutrophil function due to hyperosmolarity can be excluded.

DISCUSSION

It as been widely reported that diabetic patients have increased susceptibility to bactericidal and fungal infections which are related to impaired neutrophil function. The causes of altered neutrophil function is not fully understood.

In this study we have shown that phagocytosis of candida by diabetic neutrophils is normal at a physiological concentration of glucose and at concentrations of glucose commonly seen in the diabetic patient (10 and 20 mM). These results are in agreement with other published reports (Miller & Baker, 1972; Kaneshige *et al.*, 1982; Tater *et al.*, 1987).

The ability of neutrophils to kill candida at either 10 min or 30 min was examined, in parallel with phagocytosis, under the same metabolic conditions. Ten-minute incubations represent early oxidative phase killing, which relies on products of glucose metabolism, i.e. NADPH, whereas 30-min incubations represents bactericidal killing by oxygen-independent mechanisms, e.g. lysozyme, lactoferrin, acids, cationic proteins.

C. albicans was chosen as the test organism as it is a common pathogen seen in diabetic patients; also, the organisms are killed mainly by oxygen-dependent mechanisms (Ballart *et al.*, 1987). It is this mechanism of killing which in this study is of most interest.

An increase in glucose to 20 mM did not influence the ability of control neutrophils to kill candida at either 10 min or 30 min. However, in patients with diabetes, 20 mM glucose significantly inhibited killing within the first 10 min. The ability to kill candida by the diabetic group was only 11·26% lower than the control group at 5 mM, but at 30 mM killing was 32·4% lower in the diabetic group.

By 30 min the oxidative burst has slowed down and non-oxidative killing methods have taken over. After a 30-min incubation period the diabetic group were able to kill candida to the same extent as the controls, which suggest that killing by non-oxidative means is not effected *in vitro* by the metabolic burst. As in a previous study (Wilson & Reeves, 1986), it is the oxidative phase of killing which is affected by metabolic disturbance.

The impairment of oxidative killing seen in diabetic neutrophils when glucose concentrations are elevated to 20 mM is 21·69%. This is in the same range as the 24·4% reduction in the respiratory burst (using superoxide production as an index of the burst) when glucose concentrations were raised from 5 to 20 mM, as demonstrated in this laboratory (Wilson *et al.*, 1988), which suggests that a reduction in superoxide anions is responsible for the depressed killing seen in the present study.

Statil was added to all the *in vitro* incubations at a final concentration of 11 μM. In a study by Price *et al.* (1988) average plasma levels of patients taking a 300 mg/day oral dose of statil were of this order.

Statil had no effect on phagocytosis, killing of candida by the control group and on the latent non-oxidative phase of killing in the diabetic group. When statil was added to the diabetic

neutrophils and incubated for 10 min, previously depressed candida killing levels were raised to levels similar to those of the controls. Statil has also been shown to increase superoxide output in diabetic neutrophils (Wilson *et al.*, 1988).

In another study neutrophil aldose reductase activity has been measured and shown to correlate with the oxidative burst (submitted for publication). The higher the aldose reductase activity, the greater the suppression of superoxide anion production when glucose concentrations were raised. These data taken into conjunction with the data presented here, have led us to suggest that aldose reductase activity is responsible for the decrease in oxidative killing ability seen in diabetic patients. At physiological concentrations of glucose (5 mM) aldose reductase activity is relatively low, but as substrate concentrations increase the activity increases, thus utilizing higher concentrations of NADPH. NADPH oxidase and aldose reductase have similar K_m values for NADPH, with the latter having the more favourable K_m (Klebanoff & Clark, 1978). Values are 40 μM for NADPH oxidase and 20–45 μM for aldose reductase. Therefore, from the kinetics it is possible that these two enzymes compete for NADPH.

The presence of the polyol pathway in the neutrophil has been confirmed by the detection of sorbitol (Wilson *et al.*, 1987). Although many groups have used sorbitol estimations as a measure of glucose flux through the polyol pathway, estimations of this kind are not useful when examining aldose reductase activity. Neutrophil membranes are not permeable to sorbitol, therefore intracellular sorbitol levels reflect an equilibrium between its formation by aldose reductase and depletion by sorbitol dehydrogenase.

The pentose phosphate pathway (PPP) is important in the oxidative killing mechanism. It provides NADPH for the reduction of molecular oxygen to superoxide anions by NADPH oxidase. PPP activity in the diabetic neutrophil has been shown to be normal (Esmann, 1968) with no effect on increasing glucose concentrations or in the presence of aldose reductase inhibitors (Tebbs, Wilson & Gonzalez, 1990). Hence, NADPH production is not altered by the changing metabolic conditions in the diabetic neutrophil or by the *in vitro* addition of aldose reductase inhibitors and is therefore not responsible for the depressed oxidative killing seen in some diabetic patients.

If indeed increased aldose reductase activity is responsible for the poor response of diabetic neutrophils to infection, the mechanisms regulating aldose reductase activity merit further investigation.

Although impaired neutrophil function in diabetes is probably induced by multiple mechanisms, the results suggest that there is impairment in oxidative killing caused by increased activity of the polyol pathway.

Aldose reductase inhibitors may play a role in enhancing neutrophil function in the presence of infection in poorly controlled diabetic subjects and therapeutic administration of these drugs may shorten the episode of infection and reduce the associated morbidity.

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