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THE EFFECT OF NITRONE SPIN TRAPPING AGENTS ON RED CELL GLUCOSE METABOLISM

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The nitrone spin trapping agents, 5,5-dimethyl-1-pyrroline-N-oxide and N-t-butyl-α-phenyl-nitrone, affect the metabolism of glucose by red cells.

Both nitrone spin trapping agents have a dose-dependent inhibitory effect on the metabolism of glucose via the hexose monophosphate pathway. The formation of lactate and pyruvate via the Embden-Meyerhoff pathway in red cells is not significantly affected by treatment with 5,5-dimethyl-1-pyrroline-N-oxide, whereas, treatment with N-t-butyl-α-phenylnitrone supresses pyruvate and stimulates lactate formation.

These results suggest that nitrone spin trapping agents inhibit the hexose monophosphate pathway in red cells. Since the stimulation of the flux of glucose oxidised via this pathway is thought to be important in the ability of red cells to respond to oxidative stress, the treatment of red cells with spin trapping agents appears to inhibit the cellular protective (antioxidant) response.

The use of nitrone spin trapping agents in the study of red cells under oxidative stress (imposed by the spontaneous autoxidation of metabolites or by drug-induced processes) is predicted to exaggerate the degree of oxidative damage by virtue of the inhibitory effort of nitrone spin traps on the hexose monophosphate shunt.

Key words: Spin trap, red cell, glycolysis, hexosemonophosphate shunt

1. INTRODUCTION

The electron spin resonance (e.s.r.) technique of spin trapping[1] has proven beneficial in the investigation of free radical-mediated processes in biological systems[2]. Nitrone spin trapping agents have been used to monitor free radical formation following treatment of red cell suspensions with several xenobiotic and pharmacologic compounds.

The haemolytic reactions of hydrazine derivatives have been studied by spin trapping techniques. Phenyl radical production has been demonstrated during phenylhydrazine-induced haemolysis[3,4]; spin trapping agents were also found to inhibit the oxidative phenomena and lysis associated with the haemolytic reaction[5]. Similar studies have demonstrated free radical production in red cell suspensions, treated with hydrazine, l-acetyl-2-phenyl-hydrazine[6] and hydralazine[7].

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The role of t-butoxy radicals in t-butyl hydroperoxide-induced lipid peroxidation and haemoglobin oxidation in red cells, has been elucidated by spin trapping studies[8]. The synchronous production of a t-butoxy radical spin adduct[8] and oxidative stress on red cell metabolism[9] implicated a major role for t-butoxy radicals in initiating the oxidative events induced by t-butyl hydroperoxide in red cells.

Free radical formation has also been observed by spin trapping in red cell suspensions treated with: i) the anti-malarial drugs primaquine and chloroquine[10], ii) the anti-neoplastic drug adriamycin[11] and 1,4-napthoquinone-2-potassium sulphonate (P.J. Thornalley and A. Stern, unpublished observations).

The use of nitrone spin trapping agents in studying drug-induced oxidative events in red cells has suggested a study of the effects on nitrone spin traps on red cell metabolism. In this report, we describe the effects on red cell metabolism upon exposure to two much-used spin trapping agents: 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and N-t-butyl-α-phenyl-nitrone (PBN).

2. MATERIALS AND METHODS

2.1 Spin trapping agents:

5,5-Dimethyl-1-pyrroline-N-oxide and N-t-butyl-α-phenyl-nitrone were purchased from Aldrich Chem. Co. Ltd., Milwaukee, WI. DMPO was purified by published methods[12] before use. PBN was used without further purification.

2.2 Red Cell Preparations:

Adult human blood was drawn daily into 3.8 per cent sodium citrate solution. After centrifugation, plasma and white cells were removed and the red cells were washed three times with phosphate-buffered saline (9 parts 0.9 per cent NaCl: 1 part 100 mM KH_2PO_4/K_2HPO_4, pH 7.4). 25 per cent (v/v) red cell suspensions were prepared for incubation with spin traps in Krebs-Ringer phosphate buffer (120 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 16.5 mM NaH_2PO_4). For incubations containing spin trapping agent the NaCl concentration in the Krebs-Ringer phosphate buffer was decreased by half the concentration of the added spin trap to maintain isotonicity of the cellular medium.

2.3 The Uptake of Spin Traps by Red Cells

25 per cent (v/v) suspensions of red cells in Krebs-Ringer phosphate buffer were incubated with 100 mM DMPO and 40 mM PBN. The incubations were sampled at t = 0 and 60 min. Samples were centrifuged for 10 min at 2000g and the supernatant assayed spectrophotometrically for DMPO (ϵ_{234} = 7700 M^{-1} cm^{-1})[13] and PBN (ϵ_{294} = 16700 M^{-1} cm^{-1})[14].

2.4 Metabolic Studies

The flux of glucose oxidised through the hexose monophosphate pathway was measured by collecting $^{14}CO_2$ released from D-[U-^{14}C] glucose (purchased from New England Nuclear, Boston, MA). (D-[U-^{14}C]-Glucose rather than D-[1-^{14}C]-glucose was used so that the entire flux — including recycling of pentoses was measured). 1 ml

samples of 25 per cent red cell suspensions, in the absence and presence of spin traps, were incubated for 1 hour in 10 ml stoppered Erhlenmeyer flasks containing a well of 0.2 ml 2 M KOH. The reaction was terminated by injecting 1.0 ml 10 per cent perchloric acid and the incubation was continued for 30 min to ensure that released $^{14}CO_2$ was trapped in the KOH. The contents of each well (KOH and trapped CO_2) were transferred into 5 ml Oxosol (National Diagnostic, Sommerville, NJ) and counted for radioactivity in a liquid-scintillation counter. Blanks were prepared by adding 0.5 ml 10 per cent perchloric acid to red cell suspensions before addition of D-[U-^{14}C]-glucose. The flux through the hexose monophosphate shunt was calculated after subtracting blank values and expressed as μ mol glucose oxidised per hour per ml packed red cells. Results presented are the means ± standard deviation of four determinations.

The concentrations of lactate and pyruvate in 25 per cent red cell suspensions with 5 mM glucose, in the absence and presence of spin traps, were determined by published methods[15]. Lactate and pyruvate assays were performed at t = 0 and after 1 hour incubation at 37°C. Data presented are the means ± standard deviations of four determinations.

2.5 Analysis of Red Cell Haemoglobin

After incubation of 25 per cent red cell suspensions with 5 mM glucose and with or without 100 mM DMPO and 40 mM PBN, at 37°C for 1 hour, the red cells were collected by centrifugation. The 0.25 ml pellets were washed once with 5 ml phosphate-buffered saline followed by lysis of the washed pellets with 4 ml water. The haemolysates were mixed thoroughly and then treated with 1 ml 100 mM sodium phosphate buffer, pH 7.4, and mixed well again. The haemolysates were centrifuged to remove membranous material and analysed for oxyhaemoglobin, methaemoglobin and non-intact haemoglobin (haemoglobin metabolites other than oxyhaemoglobin and methaemoglobin) as previously described[16]. Data presented are the means ± standard deviation of four determinations.

3. RESULTS

3.1 Uptake of Spin Trapping agents by Red Cells

DMPO and PBN are both rapidly equilibrated across the red cell membrane in Krebs-Ringer phosphate buffer, pH 7.4 and 37°C. In the time taken to sediment the red cells (10 min), the concentration of spin trap in the extracellular medium falls from the t = 0 (theoretical) concentration of spin trap in the extracellular medium, 133 mM DMPO and 53.3 mM PBN, to the expected concentration for equilibration across the red cell membrane in a 25%·(v/v) red cell suspension, 100 mM DMPO and 40 mM PBN, respectively. This indicates that there is a facile transport of DMPO and PBN into red cells. Spin trap concentrations were assayed by U.V. absorption spectrophotometry in red cell supernatants — see Materials and Methods.

3.2 Stability of Spin Trapping Agents in Red Cell Suspensions

100 mM DMPO was incubated at 37°C, with 25% suspension of red cells in Krebs-Ringer phosphate buffer, pH 7.4. After incubation for one hour, there was a decrease

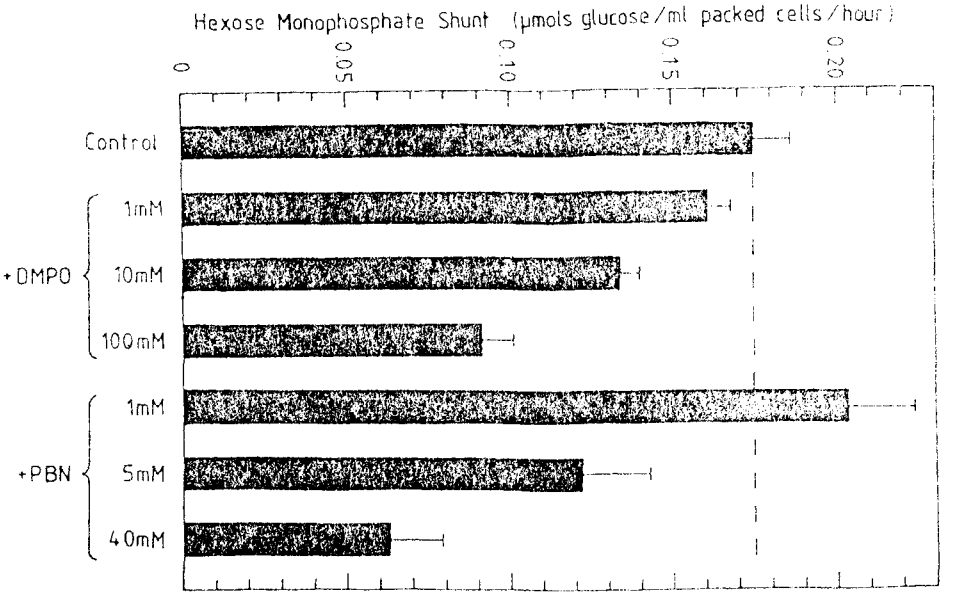

FIGURE 1 THE EFFECT OF SPIN TRAPPING AGENTS ON THE FLUX OF GLUCOSE OXIDISED VIA THE HEXOSE MONOPHOSPHATE PATHWAY IN RED CELLS.
The flux of glucose oxidised via the hexosemonophosphate pathway was measured as described in the Materials and Methods section. Data are the means ± standard deviation for four independent determinations. Red cells were incubated in Krebs-Ringer phosphate buffer, pH 7.4, and 37°C.

in the characteristic absorption band assigned to DMPO at 234 nm, corresponding to a decrease in DMPO concentration of 5 mM or 5%. This was independent of the red cell concentration and may be due to slow thermal decomposition of DMPO. PBN (40 mM) remained relatively intact throughout a similar incubation period.

3.3 The Effect of Spin Trapping Agents on the Metabolism of Glucose by Red Cells

The spin trapping agents, DMPO and PBN, both have a dose-dependent inhibitory effect on the flux of glucose oxidised via the hexose monophosphate pathway — Figure 1.

For 25% red cell suspensions incubated with 100 mM DMPO at 37°C, glucose oxidation via the hexose monophosphate pathway was inhibited by *ca.* 50%; for a similar incubation with 40 mM PBN, glucose oxidation was inhibited by *ca.* 64%. The flux of glucose oxidised via the hexose monophosphate pathway in control red cell suspensions was 0.175 ± 0.011 μ mols glucose per ml packed red cells per hour, at 37°C in Krebs-Ringer phosphate buffer, pH 7.4.

The formation of lactate and pyruvate in red cells via the Embden-Meyerhoff path-

way was also investigated — Figure 2. DMPO (1–100 mM) had no statistically significant effect on lactate and pyruvate formation in red cells. However, similar incubations with PBN (1–40 mM) gave a decrease in pyruvate formation over the entire concentration range and an increase in lactate formation for incubations containing 40 mM PBN.

3.4 The Effect of Spin Trapping Agents on Red Cell Haemoglobin

25 per cent red cell suspensions with 5 mM glucose in the extracellular medium, were incubated in Krebs-Ringer phosphate buffer at pH 7.4 and 37°C, with 100 mM DMPO and 40 mM PBN for one hour. After the incubation, there was no detectable methaemoglobin formation. Omission of the extracellular glucose in these incubations gave detectable levels of methaemoglobin formation (+5% (DMPO) and +3% (PBN) total haemoglobin). No methaemoglobin formation was detected in the control red cell suspensions. The spin trapping agents, DMPO and PBN, appear to stimulate the oxidation of oxyhaemoglobin to methaemoglobin. However, the

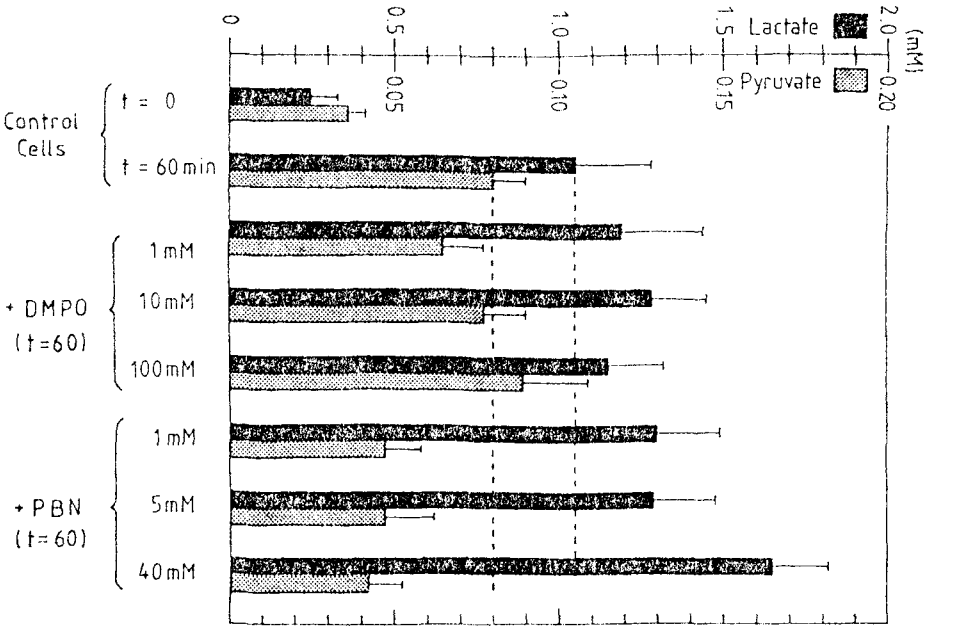

FIGURE 2 THE EFFECT OF SPIN TRAPPING AGENTS ON L-LACTATE AND PYRUVATE PRODUCTIONS IN RED CELLS.
Lactate and pyruvate concentrations are expressed in millimolar units in packed red cells. Measurements were recorded for red cells at t = 0 and t = 60 min, and for red cells treated with spin trapping agents as indicated at t = 60 min. Data are the mean ± standard deviation of four independent determinations.

methaemoglobin thereby formed appears to be within the capacity of the methaemoglobin reductase activity when glucose (at levels common to the blood plasma) is available.

4. DISCUSSION

Spin trapping agents have been used to study the mechanisms of suspected free radical-mediated processes in red cells treated with pharmaceutical agents and xenobiotic compounds[3-8,10,11]. Under such conditions, red cells often are under oxidative stress — reactive free radicals and peroxides are commonly involved in the processes. An important response of red cell metabolism to oxidative stress is thought to be the stimulation of the flux of glucose oxidised via the hexose monophosphate pathway — the hexose monophosphate shunt (HMS)[17,18].

From the results presented above it is clear that both DMPO and PBN both enter red cells easily and inhibit the native hexose monophosphate pathway activity. The observation that DMPO and PBN had no such inhibitory effect on the formation of products from the Embden-Meyerhoff pathway (lactate and pyruvate) suggests that DMPO and PBN do not inhibit glucose-6-phosphate formation in red cells (the substrate of both the hexose monophosphate and Embden-Meyerhoff pathways). Rather, DMPO and PBN inhibit the hexose monophosphate pathway at some other — as yet undisclosed — metabolic step.

The formation of methaemoglobin (above control levels) in red cells treated with DMPO and PBN suggest a mild oxidative stress is placed on the red cells by such treatment. This may be due to the lack of an adequate hexose monophosphate pathway activity to redress the oxidative effects of the spontaneous autoxidation of red cell metabolites — i.e. the initial stages of autohaemolysis.

In conclusion, the inhibition of the hexose monophosphate pathway by nitrone spin trapping agents may restrict the metabolic response of red cells to oxidative stress in spin trapping studies. It is predicted that such a use of spin traps will therefore exaggerate oxidative damage induced by the spontaneous autoxidative processes which give rise to the native hexose monophosphate shunt activity in red cells[17].

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