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Protection of Erythrocytes by the Macrophage Synthesized Antioxidant 7,8 Dihydroneopterin

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Neopterin and the reduced form, 7,8-dihydroneopterin (78NP), are pteridines released from macrophages when stimulated with γ-interferon *in vivo*. The role of 78NP in inflammatory response is unknown though neopterin has been used clinically as a marker of immune cell activation, due to its very fluorescent nature. Using red blood cells as a cellular model, we demonstrated that micromolar concentrations of 78NP can inhibit or reduce red blood cell haemolysis induced by 2,2′-azobis(amidinopropane)dihydrochloride (AAPH), hydrogen peroxide, or hypochlorite. One hundred μM 78NP prevented HOCl haemolysis using a high HOCl concentration of 5 μmole HOCl/10^7 RBC. Fifty μM 78NP reduced the haemolysis caused by 2 mM hydrogen peroxide by 39% while the same 78NP concentration completely inhibited haemolysis induced by 2.5 mM AAPH. Lipid peroxidation levels measured as HPLC-TBARS were not affected by addition of 78NP. There was no correlation between lipid oxidation and cell haemolysis suggesting that lipid peroxidation is not essential for haemolysis. Conjugated diene measurements taken after 6 and 12 hour exposure to hydrogen peroxide support the TBARS data. Gel electrophoresis of cell membrane proteins indicated 78NP might inhibit protein damage. Using dityrosine as an indicator of protein damage, we demonstrated 200 μM 78NP reduced dityrosine formation in H_2O_2/Fe^{++} treated red blood cell ghosts by 30%. HPLC analysis demonstrated a direct reaction between 78NP and all three oxidants. Two mM hydrogen peroxide oxidised 119 nM of 78NP per min while 1 mM AAPH only oxidised 50 nM 78NP/min suggesting that 78NP inhibition of haemolysis is not due to 78NP scavenging the primary initiating reactants. In contrast, the reaction between HOCl and 78NP was near instant. AAPH and hydrogen peroxide oxidised 78NP to 7,8-dihydroxanthopterin while hypochlorite oxidation produced neopterin. The cellular antioxidant properties of 78NP suggest it may have a role in protecting immune cells from free radical damage during inflammation.

Keywords: neopterin, macrophage, red blood cell, peroxidation, antioxidant, free radical, dityrosine, haemolysis

Abbreviations:7 8NP, 7,8-dihydroneopterin; AAPH, 2,2′-azobis(amidinopropane) dihydrochloride (AAPH); LDL, low density lipoprotein; RBC, red blood cell; HPLC, high performance liquid chromatography; TBARS, thiobarbitaric acid reactive species; TFA, trifluoroacetic acid

INTRODUCTION

Stimulation of human macrophages by interferon-γ (IFN-γ) causes the synthesis and secretion of the pterin; dihydroneopterin (78NP) [1,2]. The reason for this secretion is poorly understood, especially as non-primate macrophages metabolise dihydroneopterin triphosphate, the

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precursor of 78NP, to 5,6,7,8-tetrahydrobiopterin, the co-factor for nitric oxide synthase. In contrast, interferon-γ stimulation of primate macrophages causes 78NP and its oxidised form, neopterin, to be released rather than nitric oxide as in murine macrophages. At least a third of the 78NP *in vivo* is oxidised to the highly fluorescent neopterin. Measurements of plasma and urinary neopterin have been used for a number of years as a marker for immune cell activation in a wide range of clinical situations [3]. As a result, research on the physiological function of pterin synthesis has centered on the possible functions of neopterin. In *in vitro* studies, neopterin inhibits superoxide generation [4,5], stimulates *in vitro* Fenton based free radical reactions due to iron binding [6] and at high concentrations enhances the effect of tumor necrosis factor-α induced apoptosis [7].

Based on our previous studies of inhibition of lipoprotein oxidation by 78NP, we have suggested that 78NP is synthesised as a cellular antioxidant, produced by the macrophages to protect them against free radical damage during inflammation [8]. Interferon-γ acts to prime leukocyte cells before they become fully activated during an inflammatory response [9]. Macrophages require additional antioxidant defenses to function in inflammation sites due to the presence of high concentrations of superoxide, hydrogen peroxide and hypochlorite. It is likely that 78NP is responsible for some of this protection.

In support of this view, neopterin does not appear to have a significant antioxidant activity [8], whereas 78NP has been shown to scavenge superoxide anions [10,11], inhibit linoleic acid oxidation [10], inhibit H_2O_2-induced luminol chemiluminescence [12], neutralise chloride species such as chloramine-T [13,14], inhibit low density lipoprotein oxidation [8], suppress ischaemia-reperfusion injury in mice [15] and scavenge peroxyl radicals [11]. Though 78NP appears to be an effective antioxidant in these systems, the question is whether it can protect more complex cellular systems from oxidative damage. Unlike low density lipoprotein, which we have studied previously, plasma membranes have lower levels of poly-unsaturated fatty acids and consist of 50% protein.

Using porcine red blood cells (RBC), we have examined the antioxidant activity of 78NP in three different oxidising systems; a thermo-labile peroxyl radical generator, hypochlorite, and hydrogen peroxide. The latter two agents are found *in vivo* and are produced during inflammation by various leukocytes.

We used red blood cells for this research because they represent a comparatively simple cellular system, lacking both mitochondria and a nucleus. As a result, they are unable to repair damaged structures by *de novo* protein synthesis or carry out apoptosis. Catalase, which normally breaks down 92% of the hydrogen peroxide in the RBC cell [16] can be inhibited with azide treatment and the removal of glucose from the incubation media inhibits GSH formation from GSSG [17].

MATERIALS AND METHODS

All reagents used were of AR grade or better and obtained from BDH Chemicals New Zealand Limited or the Sigma Chemical Company (USA). 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) was supplied by Aldrich Chemical Company Inc. 7,8-dihydroneopterin, neopterin and 7,8-dihydroxanthopterin were obtained from Schircks Laboratories, Switzerland. Dityrosine was prepared by oxidation of tyrosine with horseradish peroxidase and purification on DEAE-sephacel (Sigma) [18]. The concentration of the purified dityrosine was determined by measuring the absorbance at 315 nm using an extinction coefficient of $E_{315} = 5080\ M^{-1}.cm^{-1}$ [19]. HOCl was purchased from Cloro-o-ogene (Petone, New Zealand). The concentration was determined using the extinction coefficient of $350\ M^{-1}cm^{-1}$ at 292 nm at pH 12 [20].

All solutions were prepared using ion-exchanged laboratory ultrafiltered water prepared in a NANOpure ultrapure water system from Barnstead/ Thermolyne (Iowa, USA). All phosphate containing buffers, including phosphate buffered saline (PBS) (160 mM sodium chloride, 10 mM sodium phosphate buffer pH 7.4), were stirred with chelex-100 resin supplied by BioRad (Richmond, USA) to remove trace heavy metal ions contaminating the phosphate salts.

Porcine red blood cells were prepared from fresh whole blood collected during slaughter at the local abattoir. Fifty mL of blood was collected into 0.5 mL of 10% EDTA for anticoagulation. The RBC were spun down and the buffy coat removed by aspiration. The red blood cells were washed twice in PBS before suspension in PBS. RBC concentration was determined by haematocrit measurement in a Heraeus haemofuge.

Oxidations were performed on 2% RBC solutions in PBS at 37 °C in the dark to prevent photo-oxidation of the 78NP. The cells were kept in suspension during the incubations by shaking in a Bioline orbital shaker (Edwards Instruments Company, Australia) at 90 rpm. The 78NP was added 10 minutes before the addition of either AAPH, HOCl or hydrogen peroxide to allow interaction with the membranes [8]. In the hydrogen peroxide oxidation experiments, RBC catalase activity was inhibited by preincubating the cells in 5 mM sodium azide for 30 minutes. This treatment was found not to have any significant affect on autolysis of RBC or RBC TBARS formation at 37°C over 14 hours.

Erythrocyte membranes (RBC ghosts) were prepared using the method of Dodge *et al.* with modifications [21]. RBC washed in PBS were suspended in 10 volumes of hypotonic buffer (10 mM sodium phosphate, pH 7.4). The membranes were pelleted by centrifugation at 23100 g for 40 minutes. The supernatant was discarded and the white pellet was suspended in 35 mL of the hypotonic buffer before pelleting at 23100 g as before. This washing step was carried out four times to remove the majority of the haemoglobin. The RBC ghosts were then stored in PBS under argon gas at 4 °C and were used within 48 hours of preparation. The protein concentration of the RBC ghost preparation was determined using the BCA method (Kit supplied by Pierce, USA) with BSA as a standard.

Haemolysis was determined by diluting 100 μL of RBC suspension to 1 mL in PBS. The sample was centrifuged at 23100 g for 10 minutes at 4 °C and the absorption of the supernatant was measured at 540 nm. The percentage haemolysis was calculated by dividing the absorption of the PBS diluted sample by the absorption of a water diluted sample.

Lipid peroxidation was determined as TBARs by HPLC on a reverse phase C18 column with fluorescence detection [22] using a Shimadzu Sil10A with cooled autosampler and RF-10Axls spectrofluorometer. Butylated hydroxytoluene (BHT) in methanol was added to all samples to prevent further lipid oxidation [23].

Conjugated dienes formation was measured by extracting the RBC lipids with chloroform/methanol, drying the extracts with nitrogen gas and measuring the 234 nm absorbance of the lipid residue dissolved in cyclohexane [24].

Dityrosine formation was measured by acid hydrolysis of the proteins followed by HPLC analysis of the hydrolysate. RBC ghost proteins were precipitated in 90% acetone at –20°C in hydrolysis vials. After centrifugation the supernatant was removed and the samples dried under vacuum. The vials were then placed into a Pico-Tag hydrolysis reaction vessel (Millipore, USA) and hydrolysed for 24 hours at 110°C with 6M HCl as previously described [25]. The hydrolysates were dried under vacuum and dissolved in 200 μl of 0.1 % trifluoroacetic acid (TFA) before transferring to autosampler vials. Twenty μL of the sample was injected onto a an Econosphere RP C-18 250 × 4.6 mm, 5 μm col-

umn (Alltech Associates Inc., USA) using the same HPLC system described for the TBAR analysis. The column was developed using a mobile phase of 2% methanol in 0.1% TFA pH 2.5 pumped at 1 mL/minute. The dityrosine was detected by fluorescence emission using a Shimadzu RF-10Axls fluorescence detector set at an excitation wavelength of 280 nm and emission detection wavelength of 410 nm. The dityrosine peaks were quantified against the dityrosine standard.

HPLC analysis of neopterin and 78NP was also performed on the Shimadzu HPLC using an Phenomenex Develosil ODS-MG-5 4.2 × 250 mm column. Ten μL of sample was manually injected and the chromatogram was developed by a mobile phase of 5% methanol in 20 mM ammonium phosphate pH 6.0 pumped at 1 mL/minute. Post column, the mobile phase first passed through a Shimadzu RF-10Axls fluorescence detector set at excitation 353 nm, emission 438 nm, to detect neopterin, then into a Shimadzu L-ECD-6A detector set at +0.6V to detect 78NP and 7,8-dihydroxanthopterin. The concentration and identity of the eluted pterin was confirmed by comparison to standards.

Statistical analysis of the data performed using the GraphPad Prism software package (GraphPad Software, Inc. USA). Significance of the data was analysed by one way and two way analysis of variance (ANOVA & MANOVA).

We found significant differences in oxidative resistance between different RBC preparation, possibly caused by variable levels of antioxidants in the pigs plasma. As the pig blood was collected during commerical slaughter, we had no control over this variable. Combining the data of a number of identical experiments, using RBC prepared from different pigs introduced large error values. As a result the data shown in the various figures is from one experiment which was representative of three identical experiments using RBCs prepared from different pig blood samples.

RESULTS

Hydrogen peroxide mediated oxidation

The addition of 2 mM hydrogen peroxide to the RBC suspension caused haemolysis to begin after a 2 hour lag period (fig. 1). The level of haemolysis increased steadily, reaching 90% after 12 hours. The addition of 10 μM 78NP to the RBC suspension caused a significant 32% decrease in haemolysis after 12 hours. The addition of higher concentrations of 78NP progressively slowed the haemolysis rate with 50 μM 78NP reducing the haemolysis level to 39% of the control value at 12 hours. 78NP did not appear to increase the lag time for haemolysis but decreased the rate of haemolysis. In separate experiments 200 uM 78NP reduced the level of hamolysis by 47 percent (data not shown).

TBARS analysis for lipid peroxidation during hydrogen peroxide exposure to RBC showed a large burst of lipid oxidation in the first two hours of incubation (fig. 2a). The increase in TBARS was not affected by the addition of 78NP. The concentration of TBARS did not significantly change after the initial rise recorded at two hours. There was no correlation between TBARS concentration and 78NP concentration or the amount of haemolysis observed. Measurement of the conjugated dienes formed during this reaction also failed to show a correlation between lipid oxidation and haemolysis (Table I). The decrease in conjugated diene measured with 50 and 100 μM 78NP was not significant.

AAPH mediated oxidation

Exposure of RBC to AAPH peroxyl radicals caused haemolysis to begin after a 2 hour lag period (fig. 3). Maximum haemolysis occurred after 14 hours incubation. 78NP addition to the RBC caused a significant inhibition of haemolysis. Ten and 20 μM 78NP failed to decrease the

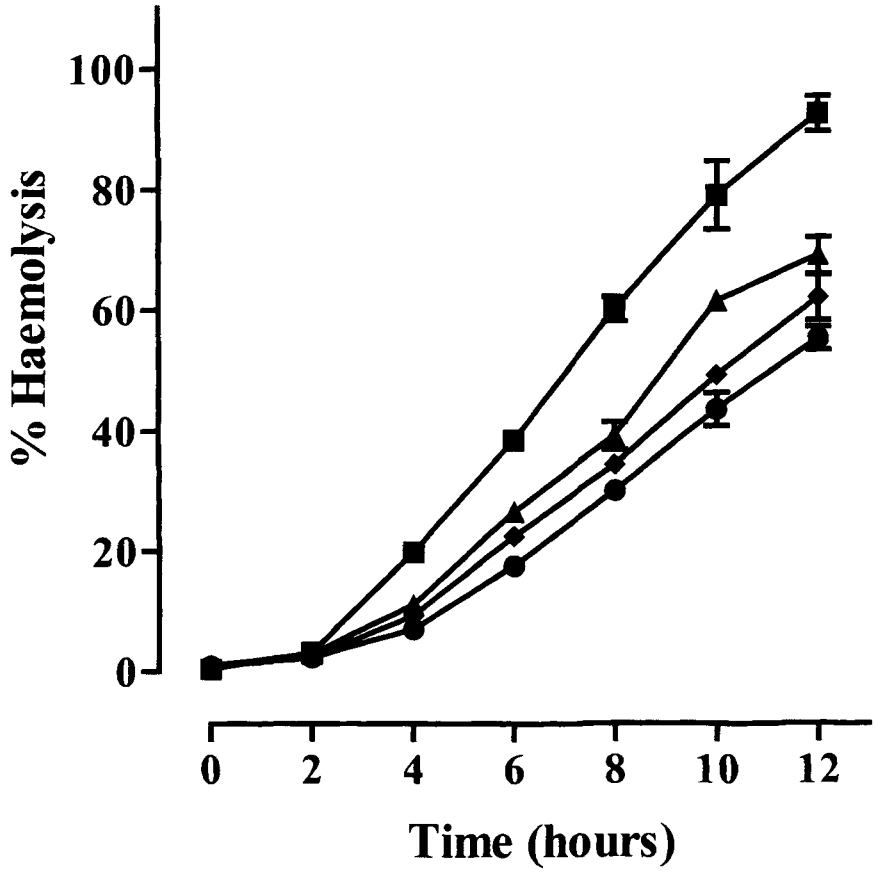

FIGURE 1 *Inhibition of H_2O_2-induced RBC Haemolysis by 7,8 dihydroneopterin.* Azide treated RBCs (2% $^{v}/_{v}$) suspensions were pre-incubated with different concentrations of 78NP at 37°C: no 78NP added (■); 10 μM 78NP (▲); 20 μM 78NP (◆); 50 μM 78NP (●). Cells were then exposed to 2 mM H_2O_2 for 14 hours. Samples were removed at 2 hour intervals and analysed for haemolysis. In the absence of hydrogen peroxide no haemolysis was observed over the 12 hour incubation. After 8 hours of incubation the level of haemolysis was significantly different to that of the control incubation for all 78NP concentrations ($p<0.001$). Data were expressed as a percentage of water diluted samples (assuming 100 % haemolysis). Graphed values are the means ± SE of two replicates. A single experiment is shown, representative of three

lag period but significantly slowed the rate during the early stage of the haemolysis. The effect of the 10 μM 78NP was no longer apparent after 10 hours with over 80% of the cells lysed. Fifty μM 78NP completely inhibited haemolysis for over 12 hours, after which some haemolysis was observed at 14 hours.

Lipid peroxidation measurements by HPLC-TBARS showed complex kinetics with an initial drop in TBARS followed by a rise in the 0, 10 and 20 uM 78NP incubations after 2 hours (fig. 2b). In contrast, the lipid peroxide concentration dropped continuously over the entire 14 hours of incubation with the 50 μM 78NP incubation. As with the hydrogen peroxide experiments, changes in the concentration of lipid peroixdes did not correlate with the onset of haemolysis (fig. 3).

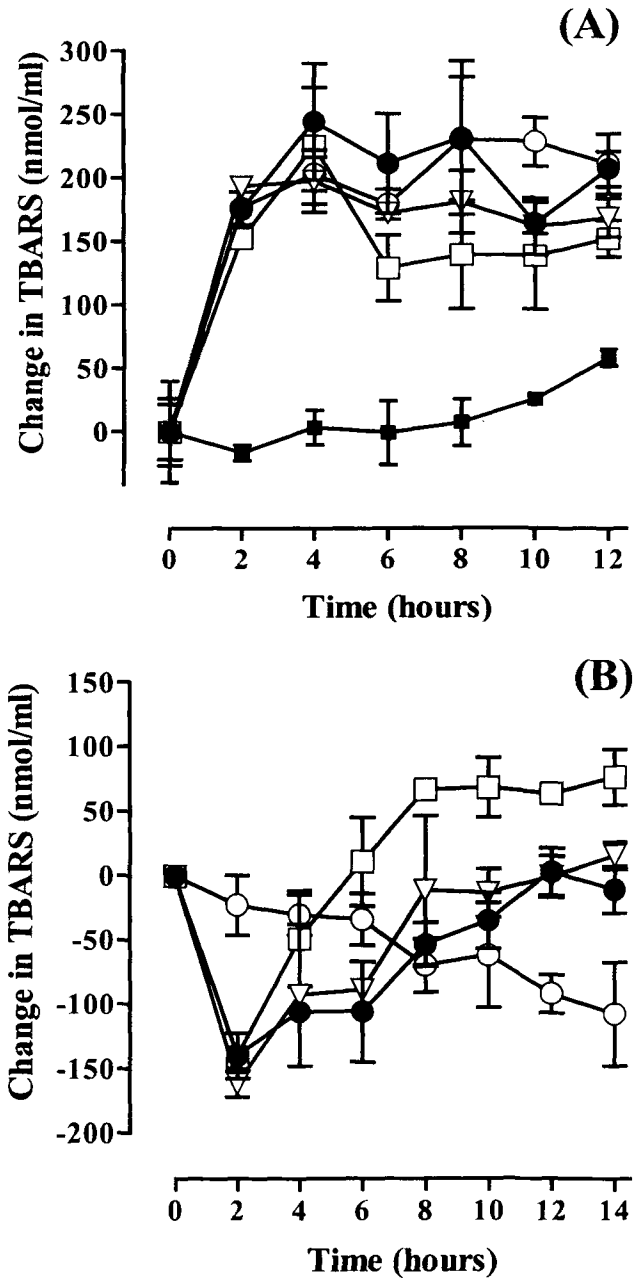

FIGURE 2 *Lipid oxidation in RBC during hydrogen peroxide or AAPH mediated haemolysis.* RBCs (2% $^{v}/_{v}$) suspensions were pre-incubated with a range of 78NP concentrations at 37°C before the addition of the oxidant. Samples were removed at 2 hour intervals and analysed for lipid oxidation by HPLC TBARS analysis. Graphed values are the means ± SE of two replicates. A single experiment is shown, representative of three. (A) 2 mM hydrogen peroxide RBC oxidation: Control, no peroxide or 78NP (■); 0 μM 78NP (●); 10 μM 78NP (▽); 20 μM 78NP (□); 50 μM 78NP (○). Mean starting concentration of TBARS for all hydrogen peroxide incubations was 338 ± 24 nmol/mL sample. None of the treatments were significantly different from the control. (B) 2.5 mM AAPH RBC oxidation: 0 μM 78NP (●); 10 μM 78NP (▽); 20 μM 78NP (□); 50 μM 78NP (○). The mean starting concentration of TBARS for all AAPH incubations was 314 ± 14 nmol/mL sample. Only the 2, 12 and 14 hour TBARS values for the 50 μM 78NP incubation are significantly different ($p < 0.05$) from the control value with no 78NP added

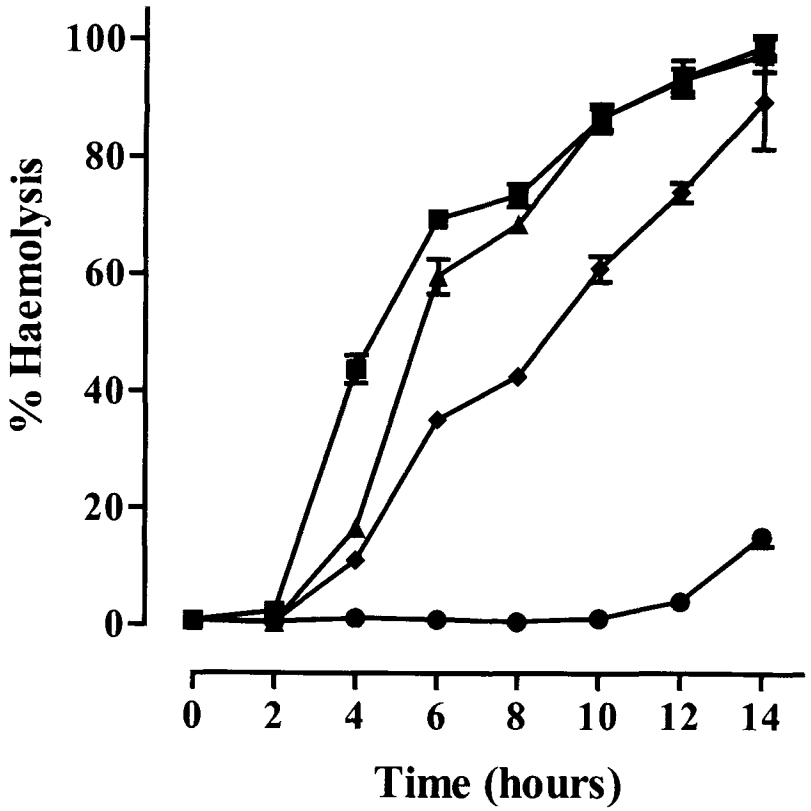

FIGURE 3 *Inhibition of AAPH-induced RBC Haemolysis by 7,8 dihydroneopterin.* RBCs (2% $^{v}/_{v}$) suspensions were pre-incubated with different concentrations of 78NP at 37°C: no 78NP added (■); 10 μM 78NP (▲); 20 μM 78NP (◆); 50 μM 78NP (●). Cells were then exposed to 2.5 mM AAPH for 14 hours. Samples were removed at 2 hour intervals and analysed for haemolysis. In the absence of AAPH no haemolysis was observed over the 12 hour incubation. Data are expressed as a percentage of water diluted samples (assuming 100 % haemolysis). Graphed values are the means ± SE of two replicates. A single experiment is shown, representative of three. From the 4 hour time point all treatments were highly significant to compared to the control ($p<0.001$) except the 10 μM 78NP which was only significant ($p<0.001$) at the 4 hour time point

TABLE I RBCs (2% suspension) were preincubated with a range of 78NP concentrations before the addition of 2 mM H_2O_2 and incubation at 37°C with shaking. After 2 and 6 hours, samples were removed for lipid extraction and conjugated diene analysis. Each value is the mean of two replicas ± standard error. The data is from a single experiment, representative of two

Condition	*Conjugated Diene Concentration, (μM)*	
	After 2 hours	*After 6 Hours*
Control RBC no H_2O_2	11.5 ± 0.6	11.7 ± 0.9
0 μM 7,8NP + 2 mM H_2O_2	11.9 ± 1.4	11.5 ± 0.7
50 μM 78NP + 2 mM H_2O_2	10.2 ± 0.8	9.8 ± 0.6
100 μM 78NP + 2 mM H_2O_2	8.3 ± 0.7	10.1 ± 0.8

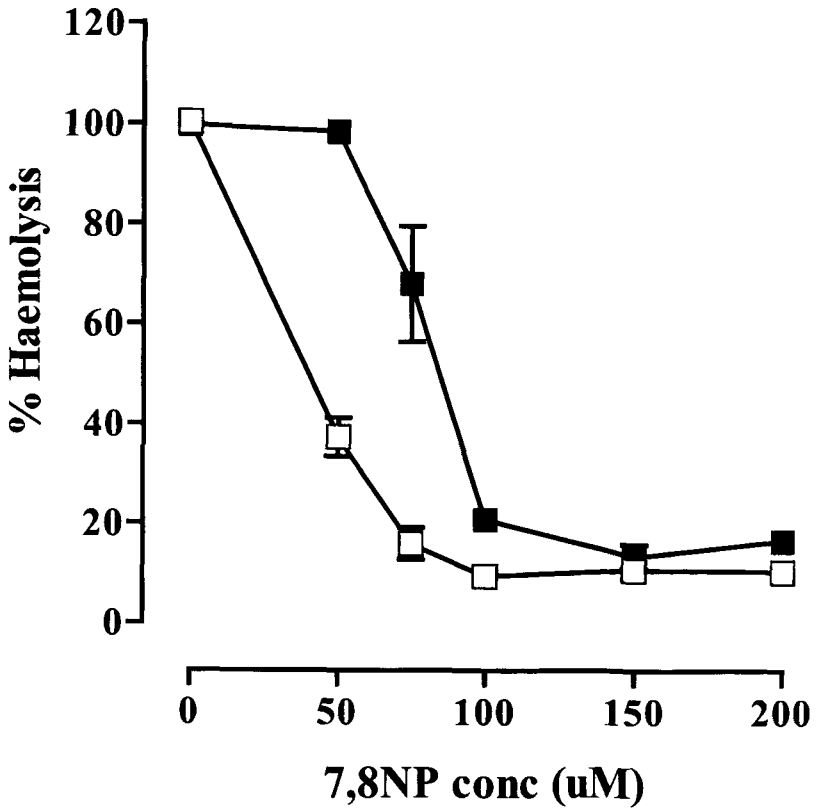

FIGURE 4 *Effect of 78NP on RBC haemolysis induced by HOCl.* RBCs were preincubated with a range of 78NP concentrations. Cells were then exposed to 5 nmoles HOCl/10^7 RBCs. Samples were removed at 30 minutes (■) and 90 minutes (□) to determine the extent of haemolysis. Data are expressed as a percentage of water diluted samples (assuming 100 % haemolysis). Graphed values are the means ± SE of two replicates. A single experiment is shown, representative of three

HOCl mediated oxidation

At a HOCl concentration of 10 nmoles/10^7 cells, 100% of the RBC were lysed after 30 minutes of incubation at 37°C (fig. 4). The addition of 50 μM 78NP to the RBC solution before HOCl addition reduced the haemolysis to 40% after 30 minutes. When the sample mixture was re-examined 90 minutes after HOCl addition, the level of haemolysis had risen to nearly 70%, indicating that the reactive processes which caused haemolysis continued to occur. 100 μM 78NP was the minimum concentration observed to completely inhibit HOCl mediated haemolysis after both 30 and 90 minutes of HOCl addition.

Protein oxidation

Denaturing SDS-PAGE analysis of RBC proteins for cross-linking or degradation during hydrogen peroxide or AAPH oxidation were inconclusive due to the high levels of globin masking any potential damage. SDS-PAGE analysis of RBC ghosts exposed to 2 mM hydrogen peroxide did show some cross-linking when 200 μM Fe^{2+} was added to the incubations. The level of cross-linking appeared to be reduced with the addition of 200 μM 78NP to the incubations (data not shown).

HPLC analysis of acid hydrolysed RBC ghost proteins after exposure to hydrogen peroxide in

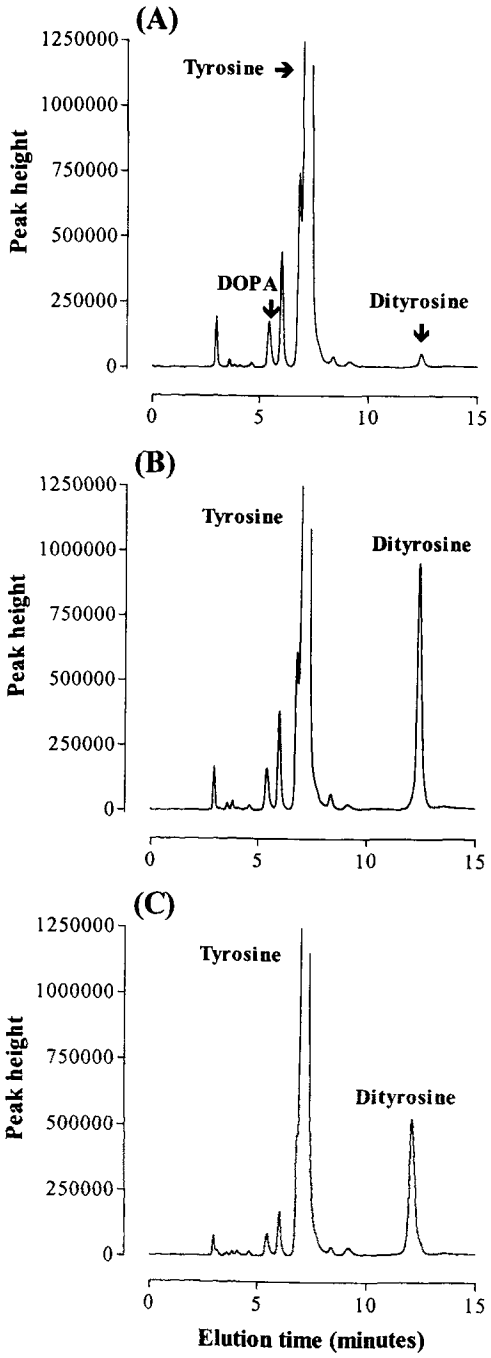

FIGURE 5 *HPLC-fluorescence chromatograms of dityrosine formation in RBC ghosts.* (A) control ghosts (no treatment added), (B) RBC ghosts treated with 200 μM Fe^{2+} + 2 mM H_2O_2 (oxidised ghosts), (C) oxidised ghosts pre-incubated with 200 μM 78NP

the presence of Fe^{2+} showed the formation of dityrosine (fig. 5), a marker for protein oxidation [18]. Pretreatment of the RBC ghosts with 50 μM 78NP did not significantly decrease dityrosine production but 100 and 200 μM 78NP reduced the dityrosine levels by 11% and 30% ($p<0.05$ and $p<0.001$ respectively) (Fig. 6).

78NP oxidation

We examined the possible reaction between 78NP and the three oxidants. HPLC analysis using fluorescence and electrochemical detection allowed the measurement of the reaction between 78NP and the three oxidants studied. Both AAPH and hydrogen peroxide exposure to 78NP in metal free phosphate buffer caused the oxidation of 78NP to 7,8-dihydroxanthopterin (fig. 7). The half-life for 50 μM 78NP at 37°C in the presence of 2 mM hydrogen peroxide was 3.5 hours which is a rate of 119 nM of 78NP oxidised per minute. Less than 5% of the 78NP was oxidised to neopterin. The half-life for 50 μM 78NP at 37°C in the presence of 1 mM AAPH was 5 hours which gives a rate of 50 nM 78NP oxidsied per minute. In contrast, the reaction with HOCl was very rapid, reaching completion in less than one minute with neopterin being the only product identified.

DISCUSSION

The majority of previous studies demonstrating 78NP antioxidant activity have used comparatively simple systems. In contrast, this study has shown that 78NP in μM concentration can protect the more complex target of a RBC from free radical damage. Using haemolysis as an experimental end point we have demonstrated 78NP is relatively effective at preventing haemolysis by HOCl and AAPH, while haemolysis by hydrogen peroxide could only be reduced (fig. 1–3). In both the HOCl and hydrogen peroxide experiments, the cells were exposed to an initial bolus

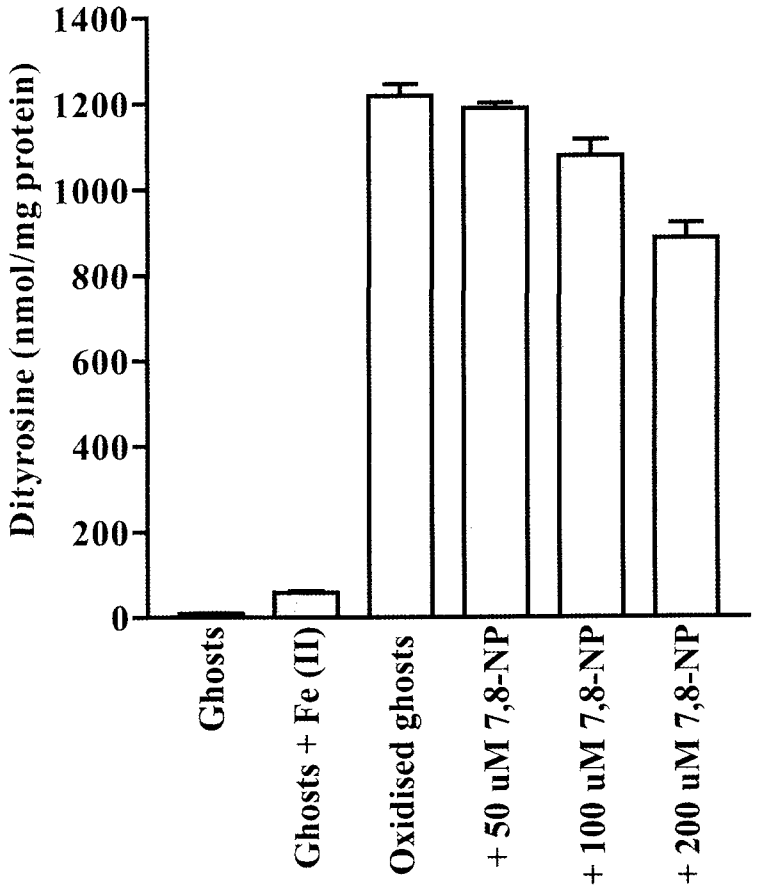

FIGURE 6 *Inhibition of dityrosine formation hydrogen peroxide treated RBC ghosts.* RBC ghosts (1.5 mg/mL protein) were pre-incubated with 0, 50, 100 and 200 μM 78NP for 10 minutes at 37°C. Ghosts were then treated with 200 μM Fe^{2+} + 2 mM H_2O_2 for 2 hours. Dityrosine formation was then measured by HPLC as described. Data are the means ± SE of 2 replicates. A single experiment is shown representative of three

of oxidant while AAPH oxidation involved the continuous generation of peroxyl radical from the AAPH [26]. The slower radical flux involved in AAPH oxidation may have allowed complete scavenging of the haemolysis-causing radicals by the 78NP. This would account for the observed lag phases of AAPH mediated RBC haemolysis (fig 3).

Hydrogen peroxide exposure also had a lag period of approximately 2 hours before haemolysis began. It is unlikely that any hydrogen peroxide was still present by the time haemolysis began. Haemolysis is a complex process which involves the loss of various key plasma proteins and a disruption of the plasma membrane [27,28]. The release of haemoglobin into the extracellular solution increases the radical flux via haem iron catalysed Fenton reactions [29]. 78NP either reacted with the hydrogen peroxide directly, reducing the initial damage to the membranes and the amount of redox active haem released, or it reacted with secondary products such as lipid peroxyl radicals. We found 78NP is oxidised by the peroxyl radicals generated by

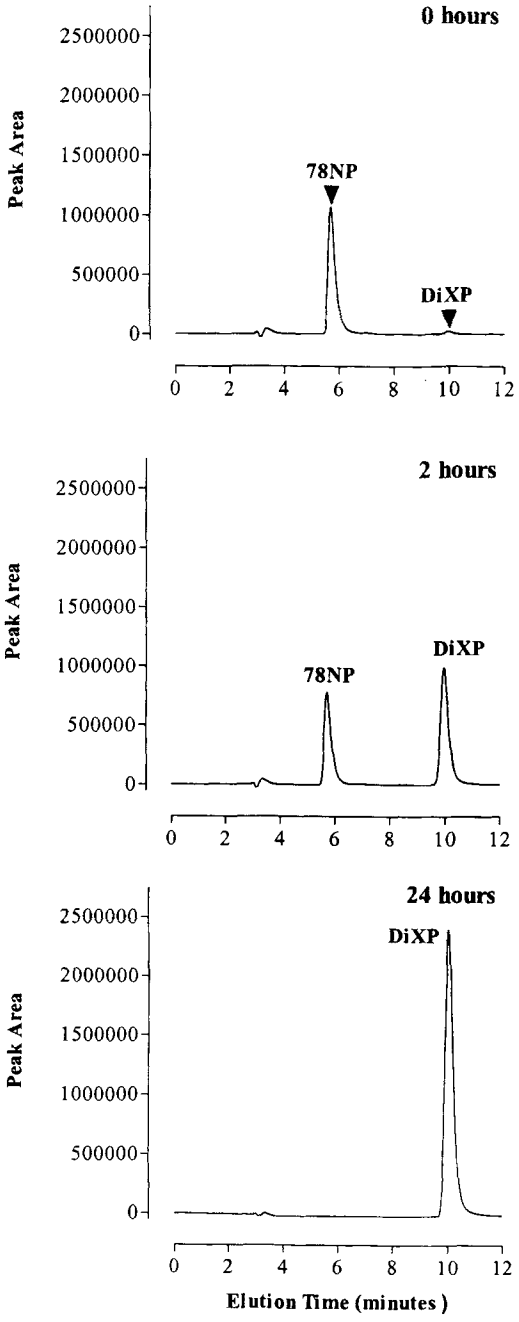

FIGURE 7 *HPLC analysis of 78NP oxidation by hydrogen peroxide.* Hydrogen peroxide was added to 50 μM of 78NP in chelexed PBS to give a final concentration of 2 mM. The mixture was incubated at 37°C in the dark with gentle swirling. At set times samples were removed for HPLC analysis with electrochemical detection

AAPH. Previous studies have reported 78NP also reacting with peroxyl radicals, during inhibition of low density lipoprotein oxidation [8] and in ESR studies [11]. For a similar mechanism to be involved in the inhibition of haemolysis by 78NP, we should have observed major changes in the concentration of lipid peroxides in the RBC during oxidation (Fig. 2a). However, HPLC-TBARS analysis only showed an initial rise in lipid peroxides after addition of hydrogen peroxide. The lipid peroxide concentration changes did not correlate with the rate or timing of haemolysis, nor was there any significant change due to varying concentrations of 78NP. Yet 78NP did inhibit haemolysis. Measurements of the conjugated dienes supported the TBARS analysis by showing no major effect of 78NP on lipid peroxidation during hydrogen peroxide mediated haemolysis, (Table I).

A more complex picture of lipid peroxidation was seen with AAPH mediated haemolysis (fig. 2b). After an initial drop in peroxides, the TBARS level slowly rose to 50 nmol/mL above the original value. The drop appears to be due to some action of the AAPH on the RBC. Controls at time zero which contained AAPH did not show this effect.

Surprisingly there is a rise in TBARS over the subsequent hours in the control, 10 and 20 μM 78NP incubation, while the TBARS level decreased with 50 μM 78NP over the entire 14 hours of the experiment. It would appear that a number of complex and competing reactions occurred between AAPH, lipid peroxides and 78NP to produce this kinetic profile.

We feel it is unlikely that lipid peroxidation is a significant process leading to haemolysis in RBC exposed to AAPH or hydrogen peroxide. Other researchers have shown similar haemolysis kinetics [30,31], but the majority of reported studies only measure lipid oxidation at a single time point. By failing to carry out time course studies as reported here, the changes in lipid peroxide levels during oxidation could be missed. Current attitudes to the importance of

lipid peroxidation has been based on studies of pure lipid systems or lipoproteins which are rich in poly-unsaturated lipids. Red blood cells have lower concentrations of unsaturated lipids and higher concentrations of membrane proteins and oxidise via different mechanisms [27,28]. However lipid peroxidation may be a significant part of the cell lysis mechanism in other cell types.

HPLC analysis showed 78NP did react directly with all three oxidants studied. Reaction with HOCl was virtually instant resulting in the formation of neopterin through the loss of the hydrogen on carbon 7 and nitrogen 8 as previously suggested [8]. We had expected a similar mechanism for the reaction between 78NP and AAPH. HPLC analysis showed less than 5% of the 78NP was oxidised to neopterin. The main reaction product was 7,8-dihydroxanthopterin (fig. 7) formed by the oxidative loss of the dihydroxypropanol group on carbon 6 of 78NP. 7,8-dihydroxanthopterin has also been reported to form during 78NP autoxidation and iron catalysed oxidation [32]. This type of direct reaction between 78NP and AAPH could explain how 50 μM 78NP was able to completely inhibit haemolysis for 12 hours. AAPH generates peroxyl radicals at a constant rate over time [26], so at sufficiently high concentrations of 78NP, all the AAPH peroxyl radicals may be scavenged, inhibiting haemolysis. The reaction between hydrogen peroxide and 78NP also generated 7,8-dihydroxanthopterin, but the reaction appears too slow to explain the effect of 78NP on hydrogen peroxide mediated haemolysis.

The apparent lack of lipid peroxide involvement in the mechanism of AAPH and hydrogen peroxide mediated haemolysis suggests the alternative view that 78NP may be affecting the rate of RBC haemolysis by preventing oxidation of key amino acids within membrane proteins.

Dityrosine has been used as a stable marker of free radical mediated protein oxidation in a number of experimental systems [33–35]. HPLC amino acid analysis showed dityrosine was formed in RBC plasma membranes as a result of Fenton reactions and that 78NP could inhibit this reaction (Fig 5&6). To our knowledge, this is the first time an antioxidant has been shown to specifically inhibit the formation of an amino acid residue oxidation product. It is possible that similar protection of plasma membrane proteins may be occurring in the more complex environment of the whole RBC. Proving this will be difficult due to the high levels of contaminating haemoglobin which would also be oxidised and cross-linked to the plasma membrane proteins [27]. Our data does show that it is possible that 78NP inhibits haemolysis by prevention of protein oxidation.

Our finding that only HOCl oxidation generates neopterin from 78NP indicates a possible source of plasma neopterin *in vivo*. HOCl is the most potent oxidant produced by neutrophils [36] and would be encountered by macrophages in an inflammation site. HOCl addition to red blood cells caused a rapid haemolysis which was completely inhibited by 100 μM 78NP addition (fig. 4). At lower 78NP concentrations, the haemolysis was slowed but not inhibited. HOCl is highly reactive and would have been completely consumed within minutes of addition to the RBC. The increased level of haemolysis seen 90 minutes after HOCl addition with the 50 and 75 μM 78NP, demonstrated a slower reaction which took time to cause haemolysis. HOCl can penetrate the red blood cell membrane and preferentially oxidise intracellular GSH [36]. However, GSH loss is not associated with HOCl-mediated haemolysis. It has been speculated that HOCl-mediated damage to membrane proteins was the initial event that led to cell haemolysis [36,37]. HOCl forms a number of reactive species on proteins including chloramines which may further damage the cell. 78NP has been shown to react with chloramines [14]. Either 78NP neutralises a portion of the HOCl reducing the initial level of damage to the RBC or it reacts with the chloramines to prevent further damage.

The actual physiological function of 78NP and neopterin *in vivo* remains unresolved. We have

shown in this report that the antioxidant activity of 78NP is sufficient to protect our model cell system of RBC. Our data and that of others [10,11] is consistent with the hypothesis that 78NP is an antioxidant within the activated macrophage.

To be an effective cellular antioxidant, micromolar concentrations of 78NP would need to be present in the macrophage cells or surrounding extracellular fluid. Plasma and urinary neopterin/78NP levels are in the high nanomolar range in clinical conditions [2,3,38], far below the concentration at which 78NP antioxidant properties are effective. Inflammation does not normally occur in the plasma but in localised sites within the tissues. Even with the diluting effects of the plasma, it is realistic to expect 78NP concentrations within inflammation sites to be in the high micromolar range and thus be an effective antioxidant. To date we are unaware of any studies on 78NP or neopterin concentration in inflammation sites or exudate.

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