FEBS Letters 347 (1994) 215–220

FEBS 14162

Alteration of the erythrocyte glutathione redox balance by *N*-acetylcysteine, captopril and exogenous glutathione

Jim Russell[a], Corinne M. Spickett[a], John Reglinski[a], W. Ewen Smith[a,*], John McMurray[b], Ibrahim B. Abdullah[b]

[a]*Department of Pure and Applied Chemistry, University of Strathclyde, Thomas Graham Building, 295 Cathedral Street, Glasgow, G1 1XL, UK*
[b]*Cardiology Unit, Glasgow Western Infirmary, Dumbarton Road, Glasgow, G11 6NT, UK*

Received 5 May 1994

Abstract

The effect of the thiol containing compounds *N*-acetylcysteine and captopril on glutathione metabolism in human erythrocytes has been investigated non-invasively using ^{1}H spin echo NMR. *N*-Acetylcysteine was found to increase the reduced form of glutathione while captopril increased the oxidized form, but no changes in the total glutathione concentration were observed. Incubation of the cells with buthionine sulphoximine to inhibit de novo glutathione synthesis did not affect the response. The results show that these compounds act by altering the redox balance of glutathione rather than by stimulating its synthesis, and that their mechanisms of action are different, and not simply due to the presence of the thiol group.

Key words: NMR; Antioxidant defence; Glutathione; *N*-Acetylcysteine; Captopril; Human erythrocyte

1. Introduction

Oxidative stress is a facet of many disease processes such as chronic heart failure [1,2], rheumatoid arthritis [3] and Graves disease [4], and it has recently been implicated in AIDS [5,6]. Chronic heart failure is further complicated by reperfusion injury, where the rapid introduction of oxygen into the ischaemic region generates active oxidants and increases tissue damage [2,7,8]. Management of these diseases includes the administration of antioxidant drugs [9] such as captopril [10] and NAC [11–14], but their mechanism of action at a cellular level is not well understood. Like glutathione, these drugs are thiol compounds, and some studies have indicated that this reactive moiety is responsible for their therapeutic action [3,4].

Oxidative stress has a profound effect on thiol balance and can result in lowered plasma thiol [15], membrane thiol [16] and erythrocyte lysate thiol [17]. The latter measurement is predominantly glutathione (~2 mM). Glutathione is a major naturally occurring antioxidant and hence the redox balance of endogenous glutathione can be used as an index of the stress on the erythrocyte [3,4]. Although glutathione responds to oxidative stress it needs to be supplemented [11] or stimulated [18,19] in chronic conditions.

NAC has been reported to stimulate the production of reduced glutathione in animal models, and it has been postulated that it is transported into the cytosol and deacetylated, thus providing more substrate for the enzyme γ-glutamylcysteine synthetase and increasing the glutathione concentration in the cells [19,20]. In contrast, captopril has been found to cause an increase in intracellular glutathione oxidation in patients who responded to therapy, suggesting that it acts by facilitating direct oxidant scavenging, rather than by stimulating glutathione production [21]. Addition of exogenous reduced glutathione to human erythrocytes has been reported to stimulate the reduction of intracellular glutathione in a manner similar to NAC [22,23].

Glutathione metabolism can be observed non-invasively in human erythrocytes using ^{1}H spin echo NMR, as this method can measure both the total concentration of glutathione and the ratio of oxidized to reduced glutathione [3,24]. In the spin echo NMR spectrum of the erythrocyte there are 4 resonances assigned to glutathione (see Fig. 1). The overall concentration of glutathione can be determined from the intensity of the glycyl (g_1) or glutamyl (g_4) resonances. The ratio of the β-cysteinyl resonance (g_2) to the glutamyl resonance (g_4) gives a measure of the glutathione oxidation-reduction status of the cell. If the g_2/g_4 ratio is high the glutathione is predominantly in the reduced form, whereas if the ratio is low the glutathione is mostly present as the disulphide [3,24]. ^{1}H spin echo NMR can therefore be used to differentiate between alterations in glutathione concentration and alterations in redox balance. Although this method can be used quantitatively [24], for kinetic studies estimates of the redox status from the g2/g4 ratio are adequate.

In this study ^{1}H spin echo NMR was used to study the mechanism of action of the drugs captopril and *N*-ace-

*Corresponding author. Fax: (44) (41) 552 5664.

Abbreviations: BSO, buthionine sulphoximine; NAC, *N*-acetylcysteine.

SSDI 0014-5793(94)00548-A

tylcysteine on the intact human erythrocyte. Glutathione signals were monitored to detect changes in glutathione concentration or redox equilibrium during treatment. γ-Glutamylcysteine synthetase was inhibited with buthionine sulphoximine to distinguish between mechanisms of action involving de novo glutathione synthesis and changes in glutathione redox balance.

2. Materials and methods

2.1. NMR experiments

All spectra were recorded on a Bruker 250 MHz Aspect 3000 spectrometer, using a Hahn spin echo pulse sequence (90°-τ-180°-τ) with a delay (τ) of 60 ms. Samples were maintained at 20°C during the data acquisition. Water suppression was achieved using a 0.5 s presaturation pulse. The spectral width was 3500 Hz and the acquisition time was 0.293 s. Each free induction decay contained 2K data points and 2048 scans were recorded for each spectrum, corresponding to a 32.5 min acquisition period. Spectra were acquired sequentially over time courses of 3 to 10 h. The g2/g4 ratio was determined by measurement of peak heights as integration cannot be carried out on negative peaks.

2.2. Preparation of erythrocytes

Human blood was obtained from healthy volunteers and was collected in lithium heparin tubes. The cells were pelleted by centrifugation at 600×*g* and the supernatant and buffy coat were removed. The erythrocytes were washed twice in PBS (154 mM NaCl, 125 mM NaH_2PO_4-NaOH pH 7.4 in D_2O) and once in saline solution (154 mM NaCl pH 7.4 in D_2O). 0.4 ml of erythrocytes were placed in a 5 mm NMR tube with 0.2 ml of saline. Cell lysates were prepared by freeze-thawing washed erythrocytes in liquid nitrogen in order to prevent dilution of the sample. 0.4 ml of lysate plus 0.2 ml of saline were used for analysis.

2.3. Treatment with thiol-containing compounds

After the acquisition of a spectrum from an untreated sample, the erythrocytes were treated in the NMR tube with thiol compounds at the following concentrations: 0.3 mM, 60 μM or 3 μM NAC (a gift from Zambon Research), 0.3 mM captopril (a gift from Bristol Myers Squibb) or 0.5 mM glutathione. Treatment with each different concentration or compound was carried out using a fresh cell sample. Similar treatments were also carried out with cell lysates in place of cell suspensions. Untreated samples were observed over the same time course as a control experiment.

2.4. Treatment of erythrocytes with BSO

After acquisition of a spectrum from untreated cells the erythrocytes were incubated with 50 μM BSO for 2 h to allow time for the inhibitor to interact with γ-glutamyl cysteine synthetase. The cells were then treated with either 0.3 mM NAC or 0.3 mM captopril.

2.5. Estimation of NAC uptake by erythrocytes

Erythrocytes were incubated with 0.3 mM NAC on the bench. Aliquots were taken at 30 min intervals and centrifuged. Sodium acetate to a final concentration of 0.5 mM was added to the supernatants and these were analysed by spin echo NMR.

2.6. Cell-free experiments with NAC

To investigate the possibility of a non-enzymatic reaction between NAC and oxidised glutathione 0.3 mM NAC was incubated with 0.3 mM diglutathione in PBS and the extent of the reaction was monitored by NMR. To determine whether glutathione reductase might be able to catalyse an interaction between NAC and diglutathione 0.3 mM NAC, 0.3 mM diglutathione and glutathione reductase (1.03 units/ml) were incubated in PBS and the reaction was observed by NMR.

2.7. Calibration of g_2/g_4 ratio

Spin echo NMR spectra of known ratios of reduced and oxidized glutathione in 5 mM sodium phosphate pH 7.4, 100 mM KCl, 5 mM MgSO4, 10 mg/ml BSA were obtained and the g_2/g_4 ratio was plotted against the ratio of reduced to oxidized glutathione.

3. Results

Fig. 1 shows a ^1H spin echo NMR spectrum of 0.3 mM NAC, and spectra of human erythrocytes before and after treatment with 0.3 mM NAC. It can be seen that under the conditions used the β-CH_2 resonances of glutathione (g_2) and NAC are coincident. As the proportion of reduced glutathione is calculated from the ratio of the g_2 and g_4 peaks, it is necessary to determine the contribution of NAC to the g_2 resonance. This was achieved by determining the ratio of the acetyl singlet at 2.1 ppm to the inverted β-CH_2 doublet at 2.8 ppm in a cell-free solution (3.5:1) and using the intensity of the acetyl peak in the intact cell spectra to calculate the correction for NAC in the g_2 peak. It was calculated from the g_2/g_4 ratio calibration curve that the proportion of reduced glutathione was approximately 75%, which is considerably lower than the values that have been obtained from invasive methods of analysis.

The intensity of the NAC acetyl resonance diminished during the time course of the NMR experiments, with the largest change occurring in the first hour (Fig. 2). As no new signals appeared in the spectrum it was concluded that this effect was unlikely to be due to metabolism of the NAC, especially as the effect also occurred in cell-free

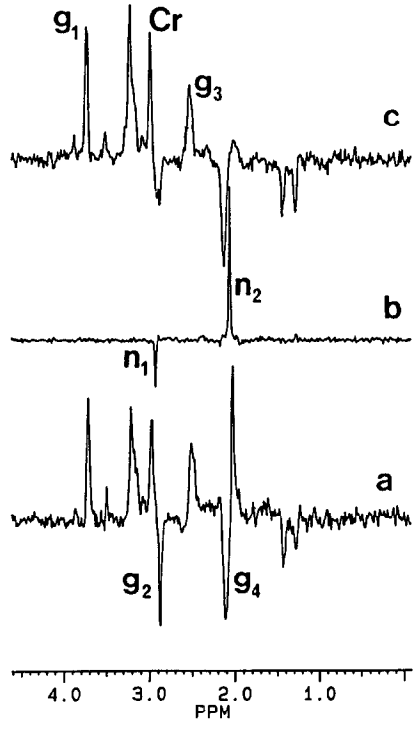

Fig. 1. ^1H spin echo NMR spectra of erythrocytes and NAC. (a) Intact erythrocytes plus 0.3 mM NAC, (b) 0.3 mM NAC and (c) intact control erythrocytes. The assignments are as follows: g_1, glycinyl CH_2; g_2, β-cysteinyl CH_2 of glutathione; g_3, glutamyl β-CH_2; g_4, glutamyl α-CH_2; n_1, b-cysteinyl CH_2 of NAC; n_2, acetyl of NAC; Cr, creatine. Spectra were acquired in 32 min and are the sum of 2048 transients. Chemical shifts are given relative to H_2O at 4.8 ppm.

solutions. It is probably caused by signal saturation; the delay time of 0.5 s is sufficient to allow complete relaxation of the intracellular species, due to the paramagnetic effect of iron present in the haemoglobin, but may not be long enough for extracellular compounds to relax fully. NMR spectra of a solution of NAC acquired using the same parameters showed that the signal saturation did not affect the ratio of the NAC resonances, and therefore that it does not present a problem for the g_2 correction.

Fig. 2 shows the effect of 0.3 mM NAC on the spectra of intact erythrocytes over time. After correction for the NAC contribution to the g_2 resonance it was found that treatment with this compound resulted in an increase in the g_2/g_4 ratio, corresponding to a shift of the intracellular glutathione to a more reduced state. This can be seen more clearly in Fig. 3, which also shows that the effect was transient, with the g_2/g_4 ratio returning to normal after approximately 3 h. The maximum increase in g_2/g_4 ratio corresponded to a shift of approximately 20% in the

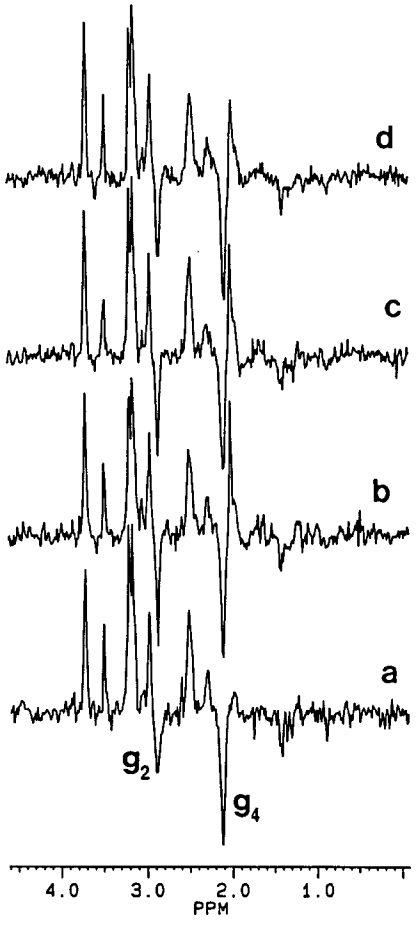

Fig. 2. Time course of treatment with NAC. Intact erythrocytes were treated with 0.3 mM NAC and ^{1}H spin echo spectra were acquired after (a) 0 min, (b) 32 min, (c) 96 min and (d) 160 min. Spectrum (a) was acquired immediately before the addition of NAC. The acquisition parameters were as described in Fig. 1. The contribution of the NAC β-cysteinyl signal to the g_2 resonance was calculated from the size of the NAC acetyl resonance.

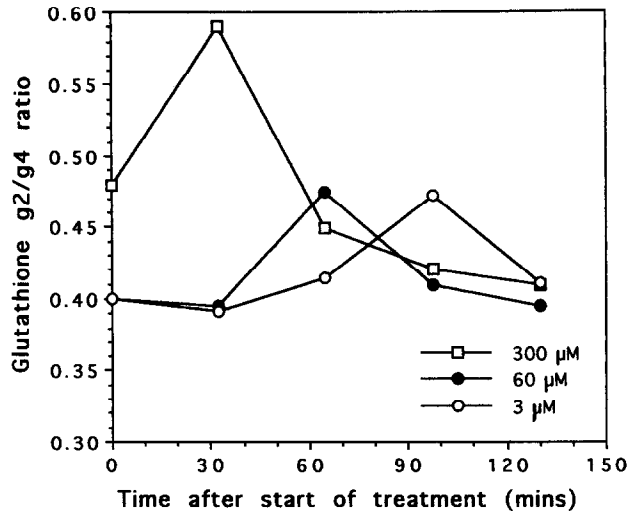

Fig. 3. Response of the g_2/g_4 ratio to NAC. The g_2/g_4 ratio was calculated from the ^{1}H spin echo spectra of intact erythrocytes treated with 300 μM, 60 μM and 3 μM NAC. The experiments were carried out on separate cell samples, hence the variation in the initial g_2/g_4 ratio. The g_2/g_4 ratio was determined by measurement of peak heights as integration cannot be carried out on negative peaks.

redox balance, from 70–90% reduced glutathione. Treatment with 2 lower concentrations of NAC (60 μM and 3 μM) resulted in a similar profile, although the length of time required to achieve the maximum effect increased with decreasing NAC concentration. Analysis of the other glutathione resonances in the spectra showed that there was no evidence of an increase in the amount of total intracellular glutathione, as the intensity of the g_1 and g_4 resonances did not change with respect to those of creatine and carnitine (Fig. 2).

The effect of treatment of intact erythrocytes with 0.3 mM captopril and 0.5 mM glutathione is shown in Fig. 4. Although the spectrum of captopril is complex, at the concentrations used most of the resonances are small compared to that of the methyl peak at 1.2 ppm and do not interfere with the measurement of the g_2/g_4 ratio. Captopril resulted in a decrease in the g_2/g_4 ratio, implying that the glutathione pool had become more oxidised (80%→45% reduced glutathione). Unlike the effect of NAC, this did not appear to be a transient effect as there were no signs of recovery after 3 h. No changes in the intensity of the g_1 or g_4 glutathione resonances were observed, suggesting that the concentration of glutathione in the cell was unaffected. Treatment with glutathione appeared to decrease the g_2/g_4 ratio, but the majority of the glutathione remains in the extracellular pool, and once this had been taken into account there appeared to be no effect on the cytosolic glutathione balance.

In order to investigate the location of NAC during treatment of erythrocytes, aliquots of the cell incubation were removed at intervals and centrifuged. Each supernatant was spiked with known concentration of sodium

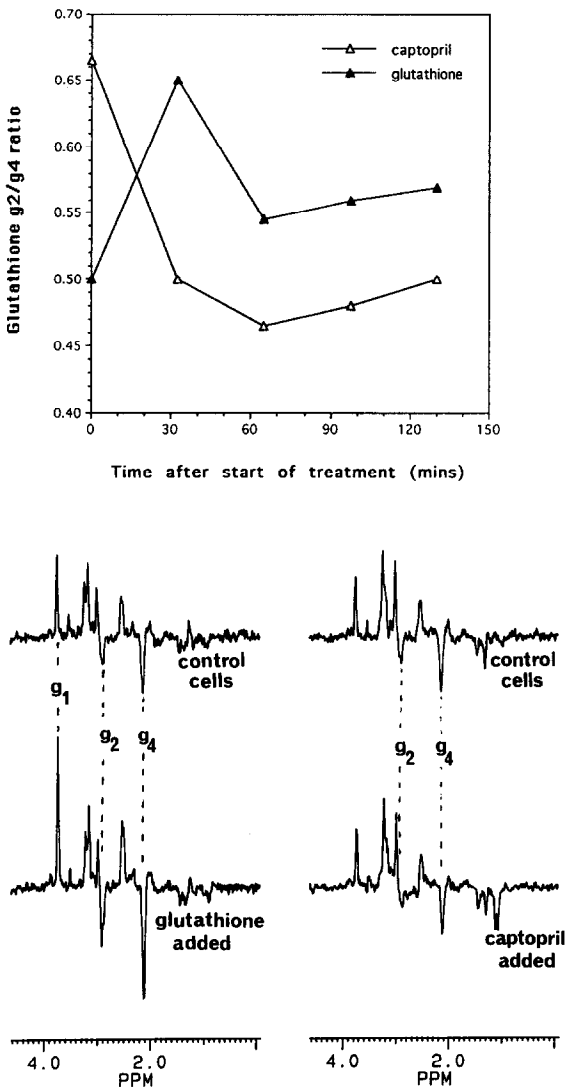

Fig. 4. Effect of treatment with captopril and exogenous glutathione. NMR spectra of erythrocytes were acquired before (control cells) and after 60 min of treatment with either 0.3 mM captopril or 0.3 mM glutathione. The graph of g_2/g_4 ratio versus time shows the effect of these compounds on the NMR spectra.

acetate as a reference, and the supernatant was then analysed by ^{1}H NMR (data not shown). There was no significant change in the ratio of the NAC acetyl peak to the reference peak over a time course comparable to those used for the in vivo NMR experiments, and it was therefore concluded that there was no significant uptake of NAC during treatment. This technique is sufficiently sensitive to show that at least 90% of the NAC remained in the extracellular space during these experiments.

The effect of BSO, an inhibitor of γ-glutamylcysteine synthetase, on the NAC- and captopril-induced changes in glutathione balance was determined in similar experiments. In order to be certain that the enzyme was substantially inhibited the cells were incubated with 50 μM BSO for 2 h; in previous work on erythrocytes 10 μM BSO was found to cause 100% inhibition after 10 min [25]. The addition of BSO resulted in a slight decrease in the glutathione concentration confirming that it was effective in inhibiting γ-glutamylcysteine synthetase. However, it was found that BSO had little or no effect on the alterations in g_2/g_4 ratio caused by the thiol-containing compounds (data not shown).

NMR spectra from a 1:1 mixture of NAC and oxidised glutathione in the absence of erythrocytes showed that no non-enzymatic thiol-transferase reaction occurred between these compounds. Experiments were also carried out to determine whether NAC could act as a substrate for the enzyme glutathione reductase in a partial equilibrium reaction of the type:

E-SH + GSSG → E-SSG + GSH

followed by

E-SSG + NAC-SH → E-SH + NAC-SSG.

This equilibrium reaction would not involve an overall redox change but the result would be to release reduced glutathione at the expense of NAC. However, incubation of NAC with diglutathione in the presence of glutathione reductase did not give any evidence for this interaction; the glutathione remained in the oxidised form and the formation of NAC-SSG was not observed. In addition it was observed that erythrocyte lysates treated with 0.3 mM NAC did not respond in the same way as intact cells, as the g_2/g_4 ratio remained unchanged during incubation. This suggests that the mechanism of action of NAC is more complex than has previously been suggested [18].

4. Discussion

NAC and captopril are thiol-containing compounds which are used to treat a variety of diseases with an oxidative aetiology such as heart failure, rheumatoid arthritis, Graves disease and AIDS [9–14]. These drugs are known to have antioxidant properties but their mechanism of action at a cellular level is not well understood. Glutathione is a major naturally occurring antioxidant, and it has been suggested that NAC might affect glutathione metabolism leading to an increase in its synthesis [19,20]. In this paper the biochemical response of human erythrocytes to these compounds has been investigated non-invasively using ^{1}H spin echo NMR to monitor simultaneously the level of glutathione and the ratio of oxidised to reduced glutathione.

The results obtained in this study show that the thiol compounds investigated did not act by increasing glutathione synthesis. The total concentration of glutathione in the cell was found to remain unchanged during treatment with NAC, but the ratio of reduced to oxidised glutathione increased (Fig. 3). Further evidence comes

from pretreatment with BSO, which did not alter the effect of NAC on the redox balance of glutathione. BSO is an inhibitor of γ-glutamylcysteine synthetase [25], which catalyses the reaction between cysteine and γ-glutamate, and hence if the action of NAC stems from increased synthesis of glutathione via this pathway, treatment with BSO should arrest the changes. It can therefore be concluded that de novo glutathione synthesis is not important in this response. It has been suggested that in some cell types NAC can be deacetylated and used as a substrate for glutathione synthesis, leading to increased levels of reduced glutathione [19,20]. It is possible that the different response obtained with erythrocytes may result from the lack of a suitable deacetylase activity in these cells, or it may reflect the fact that most previous studies reporting glutathione synthesis measured only the concentration of reduced glutathione, and were thus unable to distinguish between a redox shift and a change in total concentration. The results obtained in this investigation show clearly that NAC does not act as a cysteine delivery compound in human erythrocytes, but that there is an alternative effect involving changes in the glutathione redox balance which have not previously been reported in any cell type.

In vivo, NAC and captopril have a protective role in reperfusion injury [10,11,21]. It has been suggested that their beneficial action is due to their ability to scavenge oxygen radicals [20], thus reducing the stress on the cell and resulting in less oxidation of glutathione which is also a radical scavenger. However, in this study it was found that captopril and NAC produced opposite effects on the glutathione pool, with NAC causing a shift to a more reduced state and captopril producing a more oxidised glutathione pool, similar to the effect of penicillamine [3]. Exogenous glutathione, which has previously been reported to stimulate the reduction of intracellular glutathione in a manner similar to NAC [22,23], was found to have no effect on the endogenous glutathione. This pattern of effects on the cytosolic glutathione pool contrasts with the abilities of these compounds to scavenge the biologically important hydroxyl radical as reported in a previous study [26], and therefore it is unlikely that these compounds operate through this simplistic mechanism. Moreover, it appears that NAC and captopril act by entirely different mechanisms.

The results obtained in this study show that the effect of NAC is unlikely to be due to a direct and stoichiometric interaction with the cytosolic glutathione pool for several reasons. Firstly, it was found that the majority of the NAC remained in the extracellular fluid under the conditions of the experiment. Secondly, much lower concentrations of NAC produced a similar magnitude of effect to the higher concentration, with μmolar levels of NAC causing significant changes in the redox balance of mmolar glutathione. Thirdly, no evidence was obtained for the occurrence of either an enzymatic or non-enzymatic reaction between glutathione and NAC in experiments in vitro. Hence it appears that the effect of NAC is multiplicative or stimulatory in nature.

The difference in the mechanism of action of captopril and NAC is also indicated by the different profiles of the g_2/g_4 ratio obtained with these compounds. While captopril caused a stable oxidation of the glutathione pool, NAC resulted in a transient effect with the glutathione balance recovering by the end of the time course. The reason for the transient nature of the NAC response and the effect of NAC concentration on the profile of the response is not yet clear, but is likely to reflect important underlying features of the differential mechanisms of action of these compounds.

In conclusion, the results of this study show that the effects of NAC and captopril on glutathione metabolism cannot be due solely to a simple antioxidant property of the sulphydryl groups, as the mechanisms of action of these two compounds are clearly different. In addition NAC treatment results in a shift in the redox balance of glutathione to a more reduced state, rather than causing synthesis of glutathione [19,20]. The effect of NAC is non-stoichiometric and multiplicative in nature. We suggest that NAC and captopril act either as effectors at enzymes of glutathione metabolism such as glutathione reductase or glutathione peroxidase, or via an interaction with the cell membrane leading to a transmembrane signalling process. In a previous study evidence for action at the cell membrane of another antioxidant drug, penicillamine, has been obtained [3]. An increased understanding of the mechanism of action of these drugs is fundamental to the effective treatment of conditions of altered oxidant equilibrium such as chronic heart failure and AIDS.

Acknowledgements: We would like to thank the Science and Engineering Research Council and Zambon Research for a CASE award to J.Russell, and the SHHD for funding C.M.S.

References

[1] Ferrari, R., Alfieri, O., Currello, S., Ceconi, C., Cargnoni, A., Morzello, P., Pardini, A., Caradonna, E. and Visioli, O. (1990) Circulation 81, 201–210.
[2] Burrell, C.J. and Blake, D. (1989) Br. Heart J. 61, 4–8.
[3] Reglinski, J., Smith, W.E., Brzeski, M., Marabani, M. and Sturrock, R.D. (1992) J. Med. Chem. 35, 2134–2137.
[4] Reglinski, J., Smith, W.E., Wilson, R., Buchanan, L.M., McKillop, J.H., Thomson, J.A. (1992) Clin. Endocrinol. 37, 319–324.
[5] Kalebic, T., Kinter, A., Poli, G., Anderson, M.E., Meister, A. and Fauci, A.S. (1991) Proc. Natl. Acad. Sci. USA 88, 986–990.
[6] Staal, F.J., Roederer, M., Herzenberg, L.A. and Herzenberg L.A. (1990) Proc. Natl. Acad. Sci. USA 87, 9943–9947.
[7] Boucher, F., Pucheu, S., Coudray, C., Favier, A. and Leiris, J. (1992) FEBS Lett. 302, 261–264.
[8] Southern, P.A. and Powis, G. (1988) Mayo. Clinic Proc. 63, 381–389.
[9] Halliwell, B. (1990) Free Radical Res. Commun. 9, 1–32.

[10] Reglinski, J., Smith, W.E. and Belch, J.J.F. (1989) J. Chem. Res. 316–317.
[11] Arouma, O.I., Halliwell, B., Hoey, B.M. and Butler, J. (1989) Free Radical Biol. Med. 6, 593–597.
[12] Roederer, M., Staal, F.J.T., Ela, S.W., Herzenberg, L.A. and Herzenberg, L.A. (1993) Pharmacology 46, 121–129.
[13] Baker, D.H. (1991) Nutritional Rev. 50, 15–18.
[14] Roederer, M., Staal, F.J.T., Raju, P.A., Ela, S.W., Herzenberg, L.A. and Herzenberg, L.A. (1990) Proc. Natl. Acad. Sci. USA 87, 4884–4888.
[15] Currello, S., Ceconi, C., Cargnoni, A., Cornacchiario, A., Ferrari, R. and Albertini, A. (1987) Clin. Chem. 33, 1448–1449.
[16] Chilles, C., Mulheron, M., McCrae, F.M., Reglinski, J., Smith, W.E., Brzeski, M. and Sturrock, R.D. (1990) Ann. Rheum. Dis. 49, 668–671.
[17] Banford, J.C., Brown, D.H., Hazelton, R.A., McNeil, C.J., Smith, W.E. and Sturrock, R.D. (1982) Rheumatol. Int. 2, 107–111.
[18] Ceconi, C., Currello, S., Cargnoni, A., Ferrari, R., Albertini, A. and Visioli, O. (1988) J. Mol. Cell. Cardiol. 20, 5–13.
[19] Ogino, T., Kawabata, T. and Awai, M. (1989) Biochim. Biophys. Acta 1006, 131–135.
[20] Cotgreave, I., Moldeus, P. and Schuppe, I. (1991) Biochem. Pharmacol. 42, 13–16.
[21] Duchin, K.L., McKinstry, D.N., Cohen, A.I. and Migdalof, B.H. (1988) Clin. Pharmokinetics 14, 241–259.
[22] Cirioli, M.R., Sette, M., Paci, M. and Rotilio, G. (1990) Biochem. Int. 20, 397–403.
[23] Cirioli, M.R., Paci, M., Sette, M., De Martino, A., Bozzi, A. and Rotilio, G. (1993) Eur. J. Biochem. 215, 711–718.
[24] Rabenstein, D.L., Brown, F.F. and McNeil, C.J. (1985) Anal. Chem. 57, 2294–2299.
[25] Griffith, O.W. and Meister, A. (1979) J. Biol. Chem. 254, 7558–7560.
[26] Russell, J., Smith, W.E., Chopra, M. and McMurray, J.. Biomed. Pharm. Anal. in press.