Effect of captopril, enalaprilat and mercaptopropionyl glycine (MPG) on the oxidative activity of human isolated neutrophils

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- 1 Neutrophil NADPH oxidase produces the superoxide anion ($O_2^{\cdot-}$) anion radical from oxygen. The thiol containing ACE inhibitor, captopril has been reported to inhibit isolated NADPH oxidase. The above effect of captopril, if present in intact cells, could contribute to the ability of this drug to alleviate neutrophil-mediated tissue damage. We have, therefore, investigated the effect of captopril on the oxidative activity of intact human isolated neutrophils.
- 2 The effects of captopril on neutrophil oxidative activity were compared with those of enalaprilat (a non-thiol ACE inhibitor) and *N*-mercaptopropionyl glycine (MPG) (a simple thiol).
- 3 The oxidative response of PMA-stimulated neutrophils measured by lucigenin chemiluminescence was not affected by any of these test agents. The thiols captopril and MPG (but not enalaprilat) caused an initial delay in luminol chemiluminescence production by PMA-stimulated neutrophils.
- 4 Captopril and MPG (but not enalaprilat) increased, rather than decreased oxygen uptake, when added to PMA-stimulated neutrophils. Thiol oxidation was determined to be, at least partly, responsible for the excess oxygen uptake observed.
- 5 NADPH oxidase activity in intact neutrophils was not affected by captopril, MPG or enalaprilat. The inhibition of NADPH oxidase activity is unlikely to contribute to the therapeutic effects of captopril and other thiols.

Keywords captopril MPG thiol oxygen uptake chemiluminescence PMA

Introduction

Toxic oxygen species produced by activated neutrophils are necessary for bactericidal activity. Toxic oxygen species production begins with the conversion of superoxide ($O_2^{\cdot-}$) from oxygen, catalyzed by the NADPH oxidase situated on the outer membrane of activated neutrophils. Superoxide ($O_2^{\cdot-}$) is then partially converted to hydrogen peroxide (H_2O_2). Hydrogen peroxide is then partially converted to hypochlorous acid (HOCl), a process mediated by neutrophil myeloperoxidase [1].

The sequestration of activated neutrophils within the tissue and the subsequent production of toxic oxygen species in certain disease states, e.g. rheumatoid arthritis and experimental models of myocardial ischaemia, can cause tissue damage. Captopril, a thiol

angiotensin-converting enzyme (ACE) inhibitor, has been reported to inhibit NADPH oxidase [5] and alleviate disease activity and tissue damage under the above conditions [6–8]. These observations led us to investigate the effect of this drug on the oxidative activity of intact human neutrophils. We also studied, for comparison, the simple thiol *N*-mercaptopropionyl glycine (MPG) and the non-thiol ACE inhibitor enalaprilat.

Neutrophil superoxide/hydrogen peroxide and consequent myeloperoxidase activity oxidizes lucigenin and luminol respectively which leads to the production of chemiluminescence [9, 10] as well as oxygen uptake [1]. We used lucigenin- and luminol-derived chemiluminescence along with oxygen uptake to study

the effect of captopril on the oxidative activity of PMA-activated neutrophils. Captopril was observed to increase oxygen uptake alone, an effect that may be attributable to the oxidation of this thiol by neutrophil toxic oxygen species [11]. We, therefore, also measured thiol oxidation when captopril (and MPG) were incubated with either PMA-activated neutrophils or the toxic oxygen species, singlet oxygen.

Methods

Materials

Sodium preservative free heparin (1000 iu ml⁻¹) was obtained from Leo Laboratories Ltd, Princes Risborough, Bucks, UK. Lymphocyte separation medium 'Lymphoprep' (9.6% (w/v) sodium metrizoate, 5.6% (w/v) Ficoll-Hypaque) was obtained from Nycomed (UK) Ltd, Birmingham, UK. Enalaprilat was obtained from Merck, Sharp & Dohme Research Lab, Merck & Co Inc, Rahway, NJ, USA. All remaining compounds were obtained from Sigma, Poole, Dorset. Peripheral venous blood samples were obtained from healthy male and female volunteers.

Donors Peripheral venous blood (30 ml) was collected from 22 healthy Caucasian resting donors (male and female). Donors were aged less than 60 years and were not receiving any regular medication at the time of the study.

Isolation of neutrophils Sodium preservative-free heparin was mixed with blood (at 5 iu ml⁻¹) immediately after venepuncture. Neutrophils used for chemiluminescence experiments were separated as follows: 10 ml blood was mixed with 1 ml 6% (w/v) dextran (M Wt 500 000) in 0.9% sodium chloride and left to sediment for 45–60 min. The upper leucocyte-rich plasma layer was removed, layered onto 4 ml 'Lymphoprep' and centrifuged at 800 g for 15 min. The plasma, mononuclear cell and 'Lymphoprep' layers were discarded. The leucocyte pellet was washed and centrifuged in buffer (Balanced Salt Solution (BSS)) with 0.1% bovine serum albumin (BSA) at 300 g for 10 min and resuspended in buffer at 1×10^6 cells ml⁻¹. Neutrophils used for oxygen uptake experiments were separated by an alternative method, as this method produced the necessary higher yields of neutrophils required for these experiments. Blood (5 ml) was layered onto 4 ml 'Lymphoprep' and centrifuged at 800 g for 15 min. The plasma, mononuclear cell and 'Lymphoprep' layers were discarded and 15 ml 0.155 M NH₄Cl/0.01 M KHCO₃/0.1 mM EDTA buffer (pH 7.4) was added to lyse erythrocytes. The neutrophils were then washed and centrifuged twice, for 10 min in the above solution at 300 g. The cells were then resuspended in BSS (Balanced Salt Solution) buffer at 5×10^6 cells ml⁻¹. BSS buffer contained 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 5.5 mM (D+) glucose, pH 7.4.

The above procedures were conducted at room temperature. Neutrophils were tested within 3–4 h after isolation.

Measurement of oxygen uptake and chemiluminescence of PMA-activated neutrophils

PMA (phorbol myristate acetate) was prepared by dissolving 1 mg PMA in 5 ml dimethyl sulphoxide (DMSO) which was then dissolved in BSS buffer to 4 µg ml⁻¹ concentration.

Chemiluminescence measurements Luminol and lucigenin were dissolved at 10 mM in DMSO and BSS buffer, respectively. The 1 ml reaction mixture was prepared consisting of 4×10^5 cells ml⁻¹, 20 µM luminol or 0.25 mM lucigenin, 5 mM test drug in BSS buffer with 0.1% BSA (or BSS buffer with 0.1% BSA alone). The reaction mixture was then pre-incubated for 10 min at 37° C before 0.2 µg ml⁻¹ PMA was added. The chemiluminescence produced by the PMA-activated neutrophils was measured using a LKB Wallac 1251 Luminometer (count time 2 s, range 194–656 nm) alongside neutrophils without PMA and drug for 30 min.

Oxygen uptake measurements Oxygen uptake of neutrophils was measured, as previously described [12] using a Clark-type oxygen electrode. Neutrophil suspension (1.5–1.8 ml; $3.0\text{--}5.0 \times 10^6$ cells ml⁻¹) in BSS buffer was placed into a 3 ml sample chamber and left to equilibrate at 37° C for 5 min. BSS buffer or test drug in BSS buffer was then added and the mixture was pre-incubated for 10 min before addition of PMA. The cell concentration used for all experiments was 4.5×10^6 cells ml⁻¹. Oxygen uptake was expressed as nmol/10⁶ cells.

DTNB assay The extent of thiol oxidation was determined by measurement of the reduced -SH concentration of (a) the supernatant of 'resting' neutrophils (no PMA added) and PMA-activated neutrophils incubated with either captopril or MPG for 30 min at 37° C, (b) captopril and MPG, incubated with singlet oxygen produced from photo-illuminated rose bengal. The supernatant in (a) was extracted by filtering the neutrophil suspension through a 0.2 µm Millipore filter. The reduced -SH concentration was measured by the method of Ellman [13] using 5,5'-dithio-bis-(2-nitrobenzoic acid). Thiol concentrations of 1 mM were chosen because the Ellman assay was less sensitive to changes in -SH concentration at higher thiol concentrations.

Singlet oxygen produced from rose bengal—oxygen uptake measurements Singlet oxygen (¹O₂) is produced from the photo-illumination of rose bengal [14]. Rose bengal (0.125 mM) was added to 2.0 ml pre-warmed buffer with 0.5 mM captopril or 0.5 mM MPG or buffer alone at 37° C. The mixture was then photo-illuminated with a tungsten lamp whilst oxygen uptake was recorded for 30 min at 37° C. Oxygen uptake was expressed as nmol ml⁻¹.

Statistical analysis of results

The chemiluminescence and oxygen uptake results were analysed by three-way analysis of variance. Bonferroni multiple comparisons were also used to determine the actual time points at which captopril/MPG exerted their effect for the oxygen uptake experiments (see Figure 3).

Results

Measurement of oxygen uptake and chemiluminescence of PMA-activated neutrophils

Chemiluminescence measurements Chemiluminescence with either lucigenin or luminol was maximal at 15 min after the addition of PMA (see Figures 1 and 2). Neutrophils produced negligible chemiluminescence

Figure 1 The effect of captopril, MPG and enalaprilat (at 5 mM) on chemiluminescence (mV) by neutrophils stimulated with $0.2 \mu\text{g ml}^{-1}$ PMA, in the presence of 0.25 mM lucigenin at pH 7.4, 37°C ($n = 7 \pm \text{s.e. mean}$). ● control, □ 5 mM MPG, △ 5 mM captopril, ○ 5 mM enalaprilat.

Figure 2 The effect of captopril, MPG and enalaprilat (at 5 mM) on chemiluminescence (mV) by neutrophils stimulated with $0.2 \mu\text{g ml}^{-1}$ PMA, in the presence of $50 \mu\text{M}$ luminol at pH 7.4, 37°C ($n = 7 \pm \text{s.e. mean}$). ● control, □ 5 mM MPG, △ 5 mM captopril, ○ 5 mM enalaprilat.

essence (i.e. $< 10 \text{ mV}$ overall) in the absence of PMA with either luminol or lucigenin (data not shown).

Lucigenin Captopril, enalaprilat and MPG (at 5 mM) had little effect on the lucigenin chemiluminescence spectra of PMA-activated neutrophils (see Figure 1).

Luminol Both captopril and MPG (at 5 mM) did, however, delay the production and the decay of luminol chemiluminescence caused by PMA-activated neutrophils (see Figure 2; $P < 0.05$, 3-way analysis of variance). The effect of both thiols on luminol chemiluminescence was reproducible (data not shown). Enalaprilat did not change the luminol chemiluminescence spectra (see Figure 2).

Oxygen uptake measurements The non-thiol ACE inhibitor enalaprilat (at 5 mM) did not affect oxygen uptake by PMA-activated neutrophils. Captopril and MPG significantly enhanced oxygen uptake by PMA-activated neutrophils (see Figure 3). MPG caused significantly more oxygen uptake by PMA-activated neutrophils than captopril.

Thiol oxidation and oxygen uptake in cell supernatant from neutrophils co-incubated with captopril or MPG (at 1 mM) with or without PMA

There was negligible oxygen uptake accompanied by little or no thiol oxidation by neutrophils co-incubated with captopril or MPG in the absence of PMA, as shown in Table 1. There was also no detectable -SH in the supernatant of PMA-activated neutrophils incubated with buffer alone (data not shown).

The excess oxygen uptake of PMA-activated neutrophils in the presence of captopril and MPG (at 1 mM) was accompanied by substantial thiol oxidation. The greater enhancement of oxygen uptake shown for MPG was reflected in the greater oxidation of thiol for MPG (see Table 1).

Thiol oxidation and oxygen uptake of captopril and MPG incubated with singlet oxygen produced from rose bengal

Photo-illumination of rose bengal in buffer did not produce any oxygen uptake over an incubation time of 20 min. Thiol oxidation accompanied by oxygen uptake occurred when both captopril and MPG were incubated with singlet oxygen produced from rose bengal. For MPG, the reduced -SH decreased from 0.500 mM at 0 min to 0.254 mM ($\pm 0.042 \text{ s.e. mean}$; $n = 5$) at 7 min after the start of the reaction whilst oxygen uptake had increased from 0 to $130.434 \text{ nmol ml}^{-1}$ ($\pm 18.348 \text{ s.e. mean}$; $n = 5$). (Oxygen uptake measurements for MPG beyond this time could not be obtained as they went beyond the scale of the recorder.) For captopril, the reduced -SH concentration decreased from 0.500 mM at 0 min to 0.295 mM ($\pm 0.030 \text{ s.e. mean}$; $n = 5$) at 15 min after the start of the reaction whilst oxygen uptake had increased from 0 to $82.978 \text{ nmol ml}^{-1}$ ($\pm 15.171 \text{ s.e. mean}$; $n = 5$) (data not shown).

Figure 3 a) The effect of captopril 5 mM (Δ), b) MPG 5 mM ($\square$) and c) enalaprilat 5 mM on oxygen uptake (nmol/ 10^6 cells) by neutrophils stimulated with $0.2 \mu\text{g ml}^{-1}$ PMA at pH 7.4, 37°C ($n = 6 \pm \text{s.e. mean}$). ● control, □ 5 mM MPG, Δ 5 mM captopril, ○ 5 mM enalaprilat.

Discussion

The principal reason for this study was to determine if captopril, which can inhibit isolated NADPH oxidase, could also inhibit the respiratory burst of intact neutrophils *ex vivo* [5].

We ensured that the concentrations of captopril tested were similar to the captopril concentrations previously reported to inhibit NADPH oxidase [5].

We found that neither MPG nor captopril had much effect on neutrophil chemiluminescence in the presence of lucigenin. There was a delay in the production of luminol chemiluminescence, followed by an attenuation in its decay, in the presence of MPG and captopril. Thiols are effective scavengers of hypochlorous acid [15, 16] and this component is partly responsible for luminol chemiluminescence [9]. The thiol-dependent delay in luminol chemiluminescence could be caused by the removal of hypochlorous acid by captopril or MPG. Hypochlorous acid can also inactivate myeloperoxidase [17], the enzyme responsible for its production. The removal of hypochlorous acid by thiols could therefore sustain the production of luminol chemiluminescence, as observed in this work, by preserving the activity of myeloperoxidase. It is possible, however, that captopril and MPG produced the above effects through a non-specific interaction with the luminol alone as this effect has been previously reported for other reagents [18].

We also considered other methods of investigating the effect of captopril on the neutrophil respiratory burst to verify our results. One method would be to measure O_2^- production through the reduction of cytochrome c but this approach is not valid as thiols can directly reduce cytochrome c [15]. Another approach is to measure oxygen consumption by neutrophils. It might be anticipated that captopril and MPG would reduce oxygen consumption if thiols do inhibit isolated NADPH oxidase [5, 19]. Instead, captopril and MPG, enhanced oxygen uptake by PMA-activated neutrophils. Further investigation, however, revealed that this effect was almost certainly due to thiol oxidation rather than any primary effect upon neutrophil function. In support of this conclusion, we also found that direct oxidation of these thiols in a cell-free system also leads to increased oxygen consumption. The ability of singlet oxygen to oxidize thiols with concurrent oxygen uptake has been observed previously [20]. Similar studies by Thomas *et al.* (1988) also observed that neutrophils activated either by PMA or opsonized zymosan could oxidize thiols accompanied by excess oxygen uptake. Thomas *et al.* (1988) suggested that a number of mechanisms were responsible for this effect such as the oxidation of thiols by superoxide or auto-oxidation of thiols initiated by O_2^- (both processes were catalysed by manganese) [11]. Meanwhile, other workers [15, 16, 21] have shown superoxide and hydrogen peroxide to have little reactivity towards thiols.

NADPH oxidase activity in intact neutrophils was not inhibited by captopril in our experiments although this drug was previously reported to inhibit isolated NADPH oxidase. NADPH oxidase is situated within the cell membrane and consists of an active endo- and extrafacial component [19]. Captopril may have inhibited isolated NADPH oxidase by binding to the endofacial component in disrupted membranes, in the experiments of Egan and co-workers. Captopril may have been unable to bind to this component in

Table 1 Thiol oxidation (expressed as reduced -SH μM present 30 min after reaction start) and oxygen uptake (nmol/ 10^6 cells at 18 min after reaction start) of 1 mM captopril and 1 mM MPG incubated with neutrophils (2.5×10^6 cells mL^{-1}) with or without $0.2 \mu\text{g mL}^{-1}$ PMA, at 37°C ($n = 7 \pm \text{s.e. mean}$ for all experiments except '-PMA, +cells' where $n = 5 \pm \text{s.e. mean}$)

Condition	Compound				
	Control O_2 nmol	1 mM captopril [SH] μM	O_2 nmol	1 mM MPG [SH] μM	O_2 nmol
-PMA, -cells	—	933 ± 39	—	916 ± 25	—
-PMA, +cells	<1.00	976 ± 12	<1.00	883 ± 30	<1.00
+PMA, +cells	28.76 ± 9.04	789 ± 25	38.48 ± 16.18	517 ± 19	51.81 ± 20.57

our experiments because it was unable to pass across an intact cell membrane.

To conclude, captopril did not inhibit NADPH oxidase activity of intact neutrophils, even at high concentrations. It may be, instead, that the ability of this drug to scavenge reactive oxygen species con-

tributes, under certain conditions, to the alleviation of tissue damage produced by an active inflammatory response.

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