Suppression of oxidant production by diltiazem, nifedipine and verapamil in human neutrophils

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1. Polymorphonuclear leucocytes are a major source of toxic oxidants in vivo, causing tissue injury in certain circumstances such as ischaemia and reperfusion. Calcium ions are a key mediator in the production of oxidants by these cells. The aim of this study was to examine the effects of the widely used calcium antagonists diltiazem, nifedipine and verapamil on the production of oxidants in neutrophils.

2. Human neutrophils were freshly prepared, suspended in different luminol media and mixed with varying amounts of each calcium antagonist. They were then stimulated with either serum-opsonized zymosan or phorbol 12-myristate 13-acetate. Oxidant production was determined by three methods, namely luminol-enhanced chemiluminescence, oxygen consumption and cytochemical staining.

3. Calcium antagonists inhibited oxidant production by neutrophils. IC_{50} values for diltiazem, nifedipine and verapamil in calcium-free medium were 0.32 mmol/l (SEM 0.02), 0.27 mmol/l (SEM 0.02) and 0.24 mmol/l (SEM 0.02) respectively under zymosan stimulation, and 0.33 mmol/l (SEM 0.02), 0.26 mmol/l (SEM 0.02) and 0.13 mmol/l (SEM 0.07) respectively under phorbol 12-myristate 13-acetate stimulation. These effects were independent of the presence of Ca^{2+} in the extracellular solution. Some inhibition was also observed when the calcium antagonists were added during the course of a respiratory burst. Oxygen uptake by the cells was reduced in the presence of each calcium antagonist. Phagocytosis by the stimulated neutrophils was not affected despite inhibition of oxidant production.

4. We conclude that calcium antagonists can suppress the capacity of neutrophils to produce oxidants. This result may provide a novel explanation for the observation that delayed treatment with calcium antagonists may attenuate post-ischaemic myocardial dysfunction.

INTRODUCTION

Numerous studies have suggested that the highly toxic and reactive oxidant moieties play an important role in causing tissue damage during, for example, myocardial ischaemia and reperfusion [1]. Polymorphonuclear leucocytes are known to be a major source of oxidant production [1, 2]. Activated polymorphonuclear leucocytes can generate superoxide (O_{2}^{-}), hydrogen peroxide, hydroxyl radicals (·OH) and hypochlorous acid through NADPH oxidase and the myeloperoxidase system on the membrane surface [3–7], as described in eqns (1)–(4). The ·OH radical can probably be produced by polymorphonuclear leucocytes through interaction between nitric oxide (NO') and O_{2}^{-} radicals generated by the cells, as shown in eqn. (5) [8–10]. Generation of these oxidants depends upon the method of stimulation and involves complex regulatory mechanisms. Phorbol 12-myristate 13-acetate (PMA) stimulation is principally mediated via the reactions in eqns (1) and (2) [11, 12]. Other stimulants, e.g. serum-opsonized zymosan, may involve the reactions in all of eqns (1)–(5) [3–10].

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\begin{align*}
2O_2^- + \text{NADPH} & \rightarrow 2O_2^- + \text{NADP}^+ + H^+ \text{ (NADPH oxidase)} \quad (1) \\
2O_2^- + 2H^+ & \rightarrow H_2O_2 + O_2 \text{ (superoxide dismutase)} \quad (2) \\
H_2O_2 + Cl^- + H^+ & \rightarrow HOCl + H_2O \text{ (myeloperoxidase)} \quad (3) \\
HOCl + O_2^- & \rightarrow OH^- + O_2 + Cl^- \text{ (myeloperoxidase)} \quad (4) \\
NO^- + O_2^- + H^+ & \rightarrow OH^- + NO_2^- \text{ (homolytic cleavage)} \quad (5)
\end{align*}
\]

Calcium ions are a key mediators in the regulation of oxidant formation by polymorphonuclear leucocytes, and in maintaining optimal function of these cells [13, 14]. For example, the production of superoxide anions by stimulated neutrophils is augmented when calcium ions are available in the extracellular environment [14, 15]. Pharmacological stimulation of calcium ion movement across the neutrophil membrane, for example by means of the
calcium ionophore A23187, has been shown to produce a burst of metabolic activity culminating in the release of oxidants [15, 16]. The calcium channel-blocking drugs diltiazem, nifedipine and verapamil are widely used in the treatment of cardiovascular disorders, and are cardioprotective or cytoprotective in experimental ischaemia and reperfusion. This protection is independent of calcium channel-blocking activity [17–23] and has been referred to as 'mysterious' cardioprotection [24–26]. It has been attributed to a direct antiperoxidative action (in a similar fashion to the α-tocopherol reaction to the hydroxyl radical) [21–23]. Neutrophils exposed to pharmacological concentrations of verapamil and nifedipine exhibit reduced capacity to kill bacteria [27]. Although L-type calcium channels have not been reported to exist in neutrophil surface membrane, these experimental results call for further investigation of the effects of calcium channel blockers on neutrophil leucocytes [28]. Therefore, we have set out to explore possible effects of the calcium antagonists on the formation of oxidants in neutrophils that have been stimulated by serum-opsonized zymosan and PMA.

**METHODS**

Venous blood from healthy subjects was harvested into heparinized Vacutainers. Neutrophils were isolated by Polymorphprep™, and hypotonic lysis of contaminating red cells was performed [29]. The cells were suspended in an ice-cold PBS solution containing 0.1% glucose. The resulting cell preparations consisted of 99% polymorphonuclear leucocytes with >95% viability as shown by exclusion of 0.4% Trypan Blue dye. Wright–Giemsa stains revealed that more than 95% of the cells were neutrophils.

Luminol-enhanced chemiluminescence is a widely used method for *in vitro* determination of oxidants produced by various phagocytes [20–33]. Chemiluminescence was measured using a Luminometer 1250 (LKB) at 37°C with full scale representing 10 V. Cells were incubated for 10 min at 37°C before stimulation. Chemiluminescence was evaluated on the basis of either the peak value expressed in mV of recorder response or the total chemiluminescence, being the sum of a series of readings including peak chemiluminescence. The time to peak chemiluminescence was also noted. The data are presented as mean values of three experiments.

The oxygen content of the reaction mixtures was measured at 37°C using a Clark-type oxygen electrode (Yellow Springs Instruments Co.). The incubation conditions were the same as for the chemiluminescence in luminol medium but without luminol. The system was allowed to equilibrate for 5 min before adding any stimulant. Oxygen uptake of stimulated neutrophils in the presence or absence of calcium antagonists was recorded at 37°C for 30 min (final volume 2 ml) and then calculated from the percentage loss of completely saturated oxygen in PBS at 37°C and 1 atmosphere pressure. (The oxygen solubility in PBS at 37°C equilibrated with 21% oxygen at 1 atmosphere is 0.2 μmol/l [34].)

Neutrophils (1 × 10⁶ cells/ml) with either 0.5 mg of serum-opsonized zymosan or 10 ng of PMA were mixed with cold nitroblue tetrazolium (NBT) medium (for composition, see below) in a plastic conical tube (final volume 1 ml) in the presence or absence of calcium antagonists. The mixture was then incubated for 20 min at 37°C to allow phagocytosis and NBT reduction. The reaction was stopped by addition of one drop of 20–25% glutaraldehyde. The cell suspension was centrifuged for a few minutes at 250 g, washed in distilled water and mounted on a glass slide for light microscopy [16].

Chemicals and reagents were obtained from Sigma. Polymorphprep™ was obtained from NYcomed Pharma As, (product no. 221740). Nifedipine was solubilized in ethanol; the final concentration of ethanol in the reaction mixture was 1% (v/v). This concentration of ethanol was added to the medium used in control preparations for all experiments with nifedipine. Verapamil and diltiazem were dissolved in PBS of composition (mmol/l): 140 NaCl, 2.7 KCl, 16 Na₂HPO₄, 2.9 KH₂PO₄; pH 7.4. PMA (200 ng/ml) was first dissolved in DMSO. The final concentration of DMSO in the reaction mixture was <0.5%.

Hypochlorous acid was prepared by acidifying sodium hypochlorite with sulphuric acid to pH 6.2, using a molar absorption coefficient of 100 at 235 nm to calculate the concentration [35]. The hypochlorous acid was diluted immediately before use with PBS (composition as above). Hypochlorous acid was tested in a functional assay in which α₁-antiproteinase was incubated with elastase and residual elastase activity was measured using a chromogenic substrate (N-succinyl-Ala-Ala-p-nitroanilide) [36].

Hydroxyl radicals (·OH) were generated by the Fenton reaction in a mixture containing potassium dihydrogen phosphate–potassium hydroxide buffer (20 mmol/l, pH 7.4), ferric chloride (100 μmol/l), EDTA (104 μmol/l), hydrogen peroxide (1 mmol/l) and ascorbate (100 μmol/l). Solutions of ferric chloride and ascorbate were made up immediately before use, in de-aired water [37]. This Fenton reaction mixture was defined as the 100% stock concentration and was diluted as required, using distilled water. Hydroxyl radical generation was determined by the deoxyribose degradation method [37].

Superoxide radicals (O₂⁻) were produced by dissolving potassium superoxide in DMSO overnight and filtering before use [38]. The concentration of O₂⁻ was expressed as the concentration of potassium superoxide and was functionally measured by the reduction of NBT assay [39].

Zymosan A (20 mg) was washed in PBS once and incubated in 2 ml autologous fresh serum for 30 min
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at 37°C in a water bath. The serum-opsonized zymosan was then centrifuged and washed once in PBS. The pellet was resuspended in 2 ml of PBS (10 mg/ml) and kept at 4°C.

Each 100 ml of luminol medium contained 75 ml of PBS with glucose (as above) and 25 ml of 0.5 mmol/l luminol in 5 mmol/l sodium hydroxide, adjusted to pH 7.5. This is referred to as luminol medium I. Luminol medium II (100 ml) was made by adding 1 mmol/l calcium chloride and 1 mmol/l magnesium chloride hexahydrate to medium I. Luminol medium III (100 ml) was made by adding 65 mg of sodium azide to medium II. The final concentration of luminol in a reaction medium was 0.05 mmol/l, which is sufficient for reacting to oxidants generated by 1 x 10⁶ stimulated neutrophils.

Each 100 ml of NBT medium contained 1 mmol/l calcium chloride, 1 mmol/l magnesium chloride hexahydrate, 65 mg sodium azide and 2.5 mg NBT in glucose-containing PBS [16].

Results are expressed as means (SEM). Differences between treatments were analysed using Student’s t-test for unpaired samples.

RESULTS

Investigations into a possible direct oxidant-scavenging property of calcium antagonists

To look for a possible α-tocopherol-like antioxidative property of calcium antagonists involving direct scavenging of oxidants, we measured the chemiluminescence of chemically generated oxidants (·OH and hypochlorous acid) in the presence of increasing amounts of each calcium antagonist. The IC₅₀ values for the drugs were greater than 1 mmol/l at two concentrations of each oxidant (3% and 30% Fenton reaction mixture, and 7 and 70 μmol/l hypochlorous acid). The rate of reaction of the drugs with O₂⁻ (3 mmol/l potassium superoxide) at pH 7.2 was measured by NBT assay [37]. The relevant rate constant (Kₑ) calculated for each drug was less than (3.8 ± 0.18) x 10² mol⁻¹ l⁻¹ s⁻¹. We also used an α₁-antiproteinase activity protection assay to seek evidence for a direct antioxidative effect of the calcium antagonists. Inactivation of 0.5 μmol/l α₁-antiproteinase by 1.0 μmol/l hypochlorous acid was not affected by the presence of diltiazem or nifedipine at any concentration tested. Verapamil at a concentration of 0.5 mmol/l protected 40.2% of the activity of α₁-antiproteinase from inactivation [36, 40].

The viability of neutrophils in the presence of calcium antagonists

Calcium antagonists in high concentration have been shown to destroy cells [41]. We therefore examined cell viability. Neutrophils in either luminol medium or NBT medium were exposed to 1 mmol/l of each calcium antagonist at 37°C for 1 h.

The viability of cells remained >95% in each treatment. A viability of >95% was also found 1 h after stimulation by serum-opsonized zymosan and PMA, in the presence and absence of 1 mmol/l of each of the calcium antagonists.

Chemiluminescence

Neutrophils suspended in different luminol media (with calcium, calcium-free or with calcium and sodium azide) were mixed with either serum-opsonized zymosan or PMA. The production of oxidants was investigated by measuring the corresponding chemiluminescence. Figure 1a shows that,

![Fig. 1. Neutrophil chemiluminescence response (CL) induced by serum-opsonized zymosan (SOZ) and PMA. (a) Bar graph showing peak chemiluminescence induced by serum-opsonized zymosan and PMA in different media in the absence of any calcium antagonist. (b) Graph showing chemiluminescence response at different time intervals induced by serum-opsonized zymosan (●) and PMA (○) in luminol medium II in the absence of any Ca²⁺ antagonist. Values shown are means±SEM of three experiments. *P<0.01, comparison between different luminol media under the same stimulant. †P<0.01, comparison between different stimulants (serum-opsonized zymosan and PMA) in the same luminol medium.](image-url)
in the absence of calcium antagonist, neutrophils in medium I (calcium-free) and medium III (with calcium and sodium azide) generate about 50% and 86% less chemiluminescence, respectively, in response to either serum-opsonized zymosan or PMA stimulation than cells in medium II (containing calcium). The chemiluminescence induced by serum-opsonized zymosan was about 65% greater than that induced by PMA in all three media. The time to peak chemiluminescent response was approximately 85% longer in serum-opsonized zymosan-stimulated neutrophils than with PMA stimulation (Fig. 1b, \( P < 0.01 \)).

The effect of the calcium antagonists on the production of oxidants was studied by mixing neutrophils with these agents before stimulation. Fig. 2 shows that each calcium antagonist inhibited the production of oxidants by serum-opsonized zymosan-stimulated neutrophils, suspended in medium I, in a concentration-dependent fashion. The IC\(_{50}\) values were 0.32mmol/l (SEM 0.018), 0.27mmol/l (SEM 0.015) and 0.24mmol/l (SEM 0.016) for diltiazem, nifedipine and verapamil respectively. In medium II or III each calcium antagonist at varying concentrations resulted in similar relative depression of chemiluminescence (data not shown). Using serum-opsonized zymosan to generate oxidants, we found no significant difference in the concentration dependence of chemiluminescence inhibition between different media for each calcium antagonist. Verapamil, in varying concentrations, inhibited induced chemiluminescence by 48% more when oxidants were stimulated using PMA than when using serum-opsonized zymosan [the IC\(_{50}\) was 0.125mmol/l (SEM 0.007) for PMA compared with 0.24mmol/l (SEM 0.016) for serum-opsonized zymosan, \( P < 0.01 \)]. The percentage inhibition in the presence of varying amounts of nifedipine or diltiazem was unchanged [IC\(_{50}\) values of 0.26mmol/l (SEM 0.019) and 0.33mmol/l (SEM 0.02) respectively]. For each calcium antagonist, there was no significant difference in inhibition of PMA-induced chemiluminescence between different media.

To examine the effects of delayed treatment on chemiluminescence, each calcium antagonist was added 3 min after the initiation of oxidant production. Serum-opsonized zymosan stimulation was inhibited by 83.9%, 89.3% and 95.5% by 0.64mmol/l diltiazem, 0.54mmol/l nifedipine and 0.48mmol/l verapamil, respectively, these concentrations being twice the IC\(_{50}\) values (Fig. 3). Similar observations were made using luminol medium II and PMA stimulation (data not shown).

**Oxygen consumption**

Experiments were carried out to assess the effects of the calcium antagonists on oxygen uptake by stimulated neutrophils. Oxygen uptake was measured in the presence or absence of calcium antagonists at 37°C in an incubation system containing 110³ neutrophils/ml, luminol-free medium I, and 0.5mg of serum-opsonized zymosan. The mixture was incubated for 5 min in a final volume of 1.9ml. The neutrophils were activated by adding 0.1ml of serum-opsonized zymosan and oxygen uptake was then measured automatically. Control solutions contained 1% ethanol (v/v) for nifedipine and diltiazem and distilled water for verapamil. Values of oxygen consumption in the two controls were almost identical at 5.5nmol min\(^{-1}\) ml\(^{-1}\); this value was taken as 100%. Experiments were run in triplicate. Diltiazem, nifedipine and verapamil at concentrations corresponding to the IC\(_{50}\) reduced oxygen uptake by 57.5% (SEM 3.2), 53.7% (SEM 3.6) and 49.2% (SEM 2.9), respectively, when compared with controls.
Fig. 4. Photomicrographs of human neutrophils phagocytosing opsonized zymosan particles in suspension. (a) to (j) NBT reduction at several stages of phagocytosis (5-min intervals from time zero to 25 min), which was strongly inhibited by diltiazem (g), nifedipine (h) and verapamil (i) at concentrations twice their IC\textsubscript{50} values. When lower concentrations were used, stronger staining was observed (not shown). (j) Control (1% ethanol). All samples from (g) to (j) were at stage (j) (25-min after serum-opsonized zymosan stimulation). Slides were mounted with Apathy's mounting medium (Raymond A. Lamb) and examined under 100-fold magnification in oil.

(P<0.01). Without stimuli neutrophils consumed little oxygen.

**DISCUSSION**

We have explored the hypothesis that calcium antagonists may function as inhibitors of oxidant production by human neutrophils. The results indicate that the calcium antagonists diltiazem, nifedipine and verapamil can suppress the capacity of neutrophils to produce oxidants through mechanisms that do not depend on the presence of extracellular calcium.

Whether extracellular calcium is necessary for oxidant production in human neutrophils depends on the nature of the stimulus. Leukotriene B\textsubscript{4} does not induce superoxide production in the absence of extracellular calcium [42], but a chemotactic peptide (formyl-methionyl-leucyl-phenylalanine) [42, 43] and opsonized zymosan [11] do. The superoxide-producing activity of cells stimulated by the chemotactic peptide in the absence of calcium is about one-quarter the activity in the presence of calcium [43]. In our experiments the chemiluminescence recorded in the absence of calcium (luminol medium I) was approximately half that in calcium-containing luminol medium II (Fig. 1a). Other stimuli, such as immune complexes (both soluble and insoluble) and aggregated human gamma-globulin, can induce neutrophil chemiluminescence in the absence of extracellular calcium [32]. The mechanism of neutrophil activation by these different stimuli is diverse. Oxidant production induced by PMA and 1-oleoyl-2-acetyl-glycerol (which is independent of extracellular calcium [42]) has been shown to take place via activation of phospholipid-dependent protein kinase C [11, 12]. In the present

**Demonstration of oxygen radicals by cytochemical staining**

When neutrophils were suspended with serum-opsonized zymosan for 25 min the cells phagocytosed the particles, and the accompanying reduction of NBT stained the cells blue-purple. The development of this staining at 5-min intervals is shown in Fig. 4a–f. Diformazan deposits appeared only around the ingested particles and not in the cytoplasm or on the free areas of the cell membrane. When ingestion of a particle was not complete, diformazan appeared on the part of the plasma membrane attached to the particle. Non-ingested particles showed no reaction. After prolonged ingestion, intracellular phagosomes were deeply stained (Fig. 4g–i). In the presence of calcium antagonists at a concentration of twice their IC\textsubscript{50} values, phagocytosis did not take place (Fig. 4g–i). Higher concentrations of the agents resulted in greater inhibition.

Similar work was done for PMA stimulation. Neutrophils were suspended in NBT medium and stimulated with 10 ng/ml PMA for 20 min. NBT was reduced as a result of O\textsubscript{2} production. All cells were stained at 15 and 20 min incubation after PMA stimulation. This NBT reduction was also inhibited by the calcium antagonists in a similar way to the serum-opsonized zymosan-stimulated reaction (not shown).
study serum-opsonized zymosan and PMA were chosen to act as neutrophil stimulants. They induced oxidant production in human neutrophils both in the presence and in the absence of extracellular calcium (Fig. 1). In addition, neutrophils stimulated by serum-opsonized zymosan or PMA showed only 14% chemiluminescence in luminol medium III as the activity of myeloperoxidase was inhibited by adding sodium azide. This is in accordance with the findings of other investigators [30, 33].

Diltiazem, nifedipine and verapamil are widely used calcium antagonists that represent three chemically distinct groups [44]. They act on the cardiovascular system to provide antianginal, antiarrhythmic, antihypertensive and cardiovascular protective effects. They are also used for protection from or treatment of cerebral ischaemic injury [44]. They interfere with cell functions in mast cells [45], platelets [46] and leucocytes [47]. It is now well established that calcium antagonists not only inhibit voltage-dependent calcium channels, but also act on a variety of other mechanisms, including regulation of calmodulin, Ca\(^{2+}\)-dependent ATPases, sarcoplasmic reticulum and mitochondrial calcium channels, and they have been shown to inhibit sodium channels [48–50]. The antioxidative effect of calcium antagonists described in this paper is a new and potentially important additional property. Verapamil has been shown to inhibit superoxide production induced in neutrophils by a chemotactic peptide with an IC\(_{50}\) of 0.1 mmol/l [11, 42] and to reduce oxygen consumption with an IC\(_{50}\) of 0.1 mmol/l [41]. These researchers used a reaction mixture containing 1 mmol/l calcium chloride.

In the present study the three calcium antagonists tested were found to inhibit neutrophil oxidant production; this inhibition remained even when these agents were added during the course of oxidant production (3 min after stimulation) although the degree of inhibition was reduced. This finding was supported by a reduced oxygen consumption, and by NBT reduction with complete phagocytosis revealed by cytochemical staining. The inhibitory actions of these agents were not attributable to any reduction of cell viability – the cells were capable of generating chemiluminescence for up to 3 h on exposure to the highest concentrations of stimulant used. To date, drug-sensitive calcium channels have not been demonstrated in neutrophils [28]. This is consistent with our observation that extracellular calcium is not necessary for the 'antioxidant' effects. It is known that protein kinase C is located in the plasma membrane [45] and that NADPH oxidase is associated with the plasma membrane [51]. It is possible that the calcium antagonists may inhibit NADPH oxidase directly and so block signal transmission leading to oxidant generation. We were surprised to find that the representatives of each class of calcium antagonist tested demonstrated very similar dose–response relationships, but intracellular mechanisms involving calcium handling may be involved. Further biochemical studies are necessary to investigate these possibilities, but we are led to conclude from the present data that inhibition of oxidant production by calcium antagonist drugs is attributable to some mechanism independent of their calcium channel-blocking property.

Because high concentrations of the calcium antagonists were needed to produce the above effects, the relevance of these findings to any therapeutic actions of the drugs may be called into question. The concentration of calcium antagonists found clinically in the human plasma is about 0.1 µmol/l. However, the drugs are highly lipophilic, which could result in a much greater (up to 1000-fold) accumulation in cell membranes than in plasma [52–54]. Therefore the drugs may accumulate in relatively high concentration in neutrophil membranes and inhibit NADPH oxidase and protein kinase C. The concentration of oxidants in a microenvironment can reach 75 µmol/l or even 5 mmol/l [6, 55, 56]. The large amount of extremely reactive oxidants in a microenvironment may disarm the inhibitory property of the antagonists before their accumulation in cell membranes. This possibility was excluded in this study by using delayed treatment (Fig. 3), chemiluminescence detection of chemically generated oxidants, an NBT assay and an \(\alpha\)-antiprotease protection assay. It is possible that the drugs could be acting via a direct, \(\alpha\)-tocopherol-like antioxidative mechanism [21–23]. The compounds should have reacted with oxidants and have been oxidized if they are sufficiently sensitive to oxidant reaction and are administered after the onset of oxidant production. This may not result in any reduction in the generation of chemiluminescence as the resultant secondary oxidants may activate luminol [30–33]. In addition, they must have much greater reactive rates than luminol (0.05 mmol/l final concentration) to favour their competition for oxidant reaction. Thus, it seems implausible that the calcium antagonists are cytoprotective in vitro through a direct antioxidative action.

It is indeed 'mysterious' that even delayed treatment with calcium channel-blocking drugs may attenuate post-ischaemic dysfunction, and mechanisms other than those postulated here are likely to be important in this regard. However, when the highly lipophilic nature of the calcium antagonists is combined with their inhibitory action on oxidant production by activated neutrophils, these agents may be capable of cytoprotective effects in vivo despite delayed treatment.

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