Effects of omeprazole on neutrophil chemotaxis, super oxide production, degranulation, and translocation of cytochrome b₂₄₅

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Abstract

The effects of omeprazole on polymorphonuclear neutrophil (PMN) chemotaxis, superoxide generation, degranulation and translocation of cytochrome b₂₄₅ were investigated. Omeprazole (10^{-6} – 5×10^{-3} mol/l) reduced chemotaxis under agarose in a dose dependent manner, and the effect was irreversible. Superoxide anion generation was inhibited 50% at a concentration of 2.5×10^{-5} mol/l and completely abolished at 5×10^{-3} mol/l. Acid degraded omeprazole also inhibited O₂⁻ generation. Omeprazole did not scavenge O₂⁻ generated in a cell free xanthin-xanthine oxidase system. Degranulation by PMNs was inhibited only by omeprazole in concentrations above 10^{-4} mol/l. Translocation of cytochrome b₂₄₅, essential for generation of O₂⁻, was not affected by omeprazole. In conclusion, the anti-ulcer agent omeprazole in concentrations obtained during intravenous administration may inhibit the function of PMNs in vitro.

Omeprazole (5-methoxy-2-(((4-methoxy-3,5-dimethyl-2-pyridinyl)methyl)sulphonyl)-1H-benzimidazol) is a new anti-ulcer agent presently under extensive investigation.¹⁻⁴ Omeprazole is an inhibitor of the H⁺,K⁺-ATPase in the secretory membrane of the parietal cell^{2,3} and accumulates by a mechanism that is dependent on the acid environment.⁴ Only few other cells generate a low pH and one of these is the polymorphonuclear neutrophilic granulocyte (PMN).

Activation of PMNs by chemotactic factors or during phagocytosis is followed by metabolic activation, secretion of H⁺ into the phagolysosome, and alkalisation of the internal pH.⁵⁻⁹ The response to activation depends on a H⁺,Na⁺ exchange, phosphorylation of a membrane associated complex,¹⁰ and a membrane bound H⁺-ATPase.^{11,12} Translocation of a cytochrome b₂₄₅ from granules to the membrane initiates nicotinamide-adenine-dinucleotide phosphate (NADPH)-oxidase activity and the production of super oxide anion (O₂⁻) and oxygen radicals assumed to be important for intracellular bacterial killing and tissue damage.¹³

It is possible that omeprazole can accumulate in PMNs and affect their function. The present investigation describes the effects of omeprazole on neutrophil function (chemotaxis, degranulation), production of O₂⁻, and translocation of cytochrome b₂₄₅ from the granules to the cell membrane.

Methods

Omeprazole was donated by B Wallmark (Hässle, Mölndal, Sweden). Dextrane T500 and Percoll were purchased from Pharmacia Fine Chemicals, formyl-methionine-leucine-phenylalanine (FMLP), zymosan A, human and bovine serum albumin, ferricytochrome c (gr VI), cytochalasine B, superoxide dismutase, xanthine, xanthine oxidase, phenol-phthalate-β-gluronic acid, p-nitrophenyl phosphate NADH, sodium pyruvate, sodium hydrosulfite, piperazine-N'N-bis(2-ethane-sulfonic acid) (Pipes), EGTA, *Mycobacterium lysodieticus* cell walls, human lysozyme, Coomassie brilliant blue G-250 were purchased from Sigma Chemical Co. Polyethylene glycol 400 was purchased from Fisher Chemical Co, agarose (Seakem ME) from Marine Colloids, Isolymp from Gallard and Schlesinger, and medium 199 and Hepes from Grand Island Biological Co.

ISOLATION OF PMNS

Buffy coats from healthy donors were mixed with dextrane to sediment the erythrocytes. The cells were collected from the leukocyte rich plasma by centrifugation (100 g; 10 minutes; 4°C), resuspended in NaCl 0.15 mol/l, and PMNs were purified on Isolymp gradients (360 g; 35 minutes). Residual erythrocytes were removed by hypotonic lysis (distilled H₂O, 30 seconds). The leukocytes were washed thrice and were resuspended in Ca⁺⁺ Mg⁺⁺ containing buffer (Na: 119 mmol/l; K: 4.7 mmol/l; Ca: 0.75 mmol/l; Mg: 1.2 mmol/l; NaH₂PO₄/H₂PO₄: 15 mmol/l; glucose: 5.5 mmol/l; pH: 7.3) or in medium 199 (pH 7.3) with Hepes 25 mmol/l and human serum albumin 0.5 mg/ml. The cell suspensions contained more than 95% PMNs and more than 97% excluded trypan blue.

SOLUTIONS OF OMEPRAZOLE

Omeprazole was dissolved in polyethylene glycol 400 (final concentration: 50 mmol/l), aliquoted, and stored at -70°C. Omeprazole was thawed immediately before use, diluted with bicarbonate 0.75 mg/ml to 25 mmol/l, followed by further dilution with Ca⁺⁺ Mg⁺⁺ containing buffer (pH 7.3), and was used within 30 minutes.

Acid degradation was done by the addition of hydrochloric acid (0.01 mol/l) 0.1 ml to 0.9 ml of 10^{-5} – 10^{-3} mol/l omeprazole (37°C; 15 minutes). The pH was adjusted to 7.3 with Hepes buffer and the degraded omeprazole was kept on ice until used.

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Figure 1: Chemotactic response by polymorphonuclear neutrophils incubated with varying concentrations of omeprazole. The distance migrated is given in arbitrary units. (Values mean (SD) of four experiments.) (FMLP=formyl-methionine-leucine-phenylalanine.)

ASSESSMENT OF PMN FUNCTION

Migration

Migration and chemotaxis were studied by the agarose technique.¹⁴ Agarose (1% in medium 199; NaHCO₃: 400 µg/ml; Hepes 25 mmol/l; human serum albumin 0.5%) containing omeprazole or solvent was layered on microscopic slides. Six rows of three wells were punched (d: 2.5 mm). Chemoattractant (FMLP 10⁻⁷ mol/l; zymosan activated serum (ZAS)) or medium 199 was applied to the two lateral wells, and after 45 minutes. PMNs (5 × 10⁻⁵, 10 µl) in medium 199 were applied to the central well. The slides were incubated in a humidified atmosphere (90 minutes, 37°C, CO₂ 5%). The slides were then fixed in glutaraldehyde (2.5%; 18 hours), agarose was removed, and they were dried. The distance migrated was measured after magnification (×60) and the chemotactic response was given by the ratio between stimulated and unstimulated migration. Samples were measured in triplicate (interslide variation: 9%; intraslide variation 7%; no of experiments 12).

Generation of O₂⁻

Ferricytochrome c reduction was used to measure the generation of O₂⁻.¹⁵

PMNs in Ca⁺⁺Mg⁺⁺ buffer were incubated with omeprazole (30 minutes, 37°C) followed by the addition of cytochalasine B (2 µg/ml). After five minutes the PMNs were isolated by centrifugation, resuspended in buffer without drug,

and transferred to a cuvette with ferricytochrome c (0.2 mmol) in Ca⁺⁺Mg⁺⁺ buffer to a final volume of 1.0 ml. Incubations with appropriate concentrations of polyethylene glycol 400 served as controls. After the addition of FMLP (10⁻⁷ mol/l) the change in absorbance was followed in a dual beam spectrophotometer (Cary 15) with a reference cuvette containing ferricytochrome c, superoxide-dismutase (50 units/ml), and PMNs. The generation of O₂⁻ was calculated from the change in absorbance using a coefficient of 21 mM⁻¹ cm⁻¹.¹⁶ The results are given as the percentage of PMNs incubated with solvent.

Assessment of O₂⁻ generation in a cell free system

Scavenging of O₂⁻ by omeprazole was assessed in a xanthine-xanthine oxidase system. Reaction mixtures contained ferricytochrome c (25 µmol), xanthine (1 µmol), and xanthine oxidase (0.2 units) in phosphate buffer (0.12 mol/l, pH 7.4), omeprazole, or polyethylene glycol 400. The reaction was initiated with xanthin-oxidase and the change in absorbance at 550 nm was followed in a dual beam spectrophotometer with a reference cuvette containing superoxide dismutase (100 units). The results are given as the percentage of incubations with solvents.

Degranulation

The release of glucuronidase (EC 3.2.31) and lysozyme (EC 3.2.1.17) by PMNs activated with FMLP was used to measure degranulation.¹⁷

PMNs (1–3 × 10⁷/ml) in Ca⁺⁺-Mg⁺⁺ buffer were incubated with omeprazole or solvent (37°C; 30 minutes), cytochalasine B (2 µg/ml) was added, and after five minutes FMLP (10⁻⁷ mol/l). The reaction was stopped after 10 minutes by placing samples on ice. After centrifugation (1000 g, 10 minutes 4°C), the supernatant was assayed for enzymatic activity. Results are given as the percentage of activity released from unstimulated cells after sonication (Triton-x100 0.25%; 3 × 15 seconds, Branson Inst).

Glucuronidase was determined by the release of phenolphthalein from phenolphthalein-β-glucuronic acid (1 mmol/l in acetate buffer 0.1 mol/l; pH 4.4).¹⁷ Lysozyme was measured as the decrease in absorbance at 450 nm of *M lysodieticus* cell walls (0.2 mg/ml in phosphate buffer 0.062 mol/l; pH 6.2).¹⁸ Release of lactate dehydrogenase was followed as a marker of cell damage by the change in absorbance at 340 nm with sodium pyruvate as substrate.

SUBCELLULAR FRACTIONATION

Incubation and activation of PMNs

PMNs in Ca⁺⁺Mg⁺⁺ buffer (5–10 × 10⁸ cells) were incubated (30 minutes, 37°C) with omeprazole (2.5 × 10⁻⁴ mol/l) or solvent followed by activation with FMLP (10⁻⁷ mol/l; 15 minutes). Unstimulated PMNs were kept as control. The reaction was stopped with ice cold buffer; PMNs were recovered by centrifugation and resuspended in relaxation buffer¹⁹ (KCl 100

TABLE 1 Effect of degraded omeprazole on the chemotactic response of polymorph. The values given are percentages of untreated cells (median value of three experiments)

Concentration	FMLP 10 ⁻⁷ mol/l	ZAS
0	100	100
5 × 10 ⁻⁵ mol/l	99	81
2.5 × 10 ⁻⁴ mol/l	70	67
5 × 10 ⁻⁴ mol/l	46	53
1 × 10 ⁻³ mol/l	34	51

ZAS: zymosan activated serum. FMLP=formyl-methionine-leucine-phenylalanine.

mmol/l, NaCl 3 mmol/l, MgCl₂ 3.5 mmol/l, Pipes 10 mmol/l, pH 7.3).

Disruption of PMNs

PMNs in 8–10 ml relaxation buffer were pressurised with N₂ (300 psi; 30 minutes; 4°C) in a nitrogen bomb (Artisan, Waltham MA, USA) under stirring for the first 15 minutes. The cavitate was collected dropwise into EGTA (pH 7.4 final concentration 1 mmol/l). Unbroken cells (25–35%) and nuclei were removed by centrifugation (500 g, 15 minutes; 4°C).

Fractionation on Percoll gradients

Fractionation was performed essentially as previously described.¹⁹ The supernatant from the cavitate was layered on a discontinuous gradient of 9 ml Percoll (density 1.05 g/ml) layered on 9 ml with density 1.12 g/ml. After centrifugation (49 000 g, 20 minutes; 4°C; Beckman TI-60) three bands were visible and could be recovered by pipetting. Percoll was removed by centrifugation (210 000 g; Beckman TI-50) and the material was resuspended in phosphate buffered saline.

Spectroscopy

Absorption spectra (440–600 nm; Cary 15) were measured after addition of triton x-100 (0.2%). The samples were divided and solid sodium hydrosulphite (1–2 mg) was added to the sample cuvette. Cytochrome b₋₂₄₅ was quantitated from the 559 nm peak using an absorption coefficient of 21.6 mM⁻¹ cm⁻¹,²⁰ and myeloperoxidase from the 472 nm peak using an absorption coefficient of 75 mM⁻¹ cm⁻¹.²¹

Miscellaneous

Alkaline phosphatase (EC 3.1.3.1) was measured with p-nitrophenyl-phosphate as substrate (1 mg/ml) in 50 mmol/l sodium barbital buffer with MgCl₂, pH 10.5.²²

Protein was measured as described by Bradford²³ with bovine serum albumin as standard.

Results

CHEMOTACTIC FUNCTION

Preincubation with omeprazole reduced the FMLP chemotactic response in a concentration dependent fashion (Fig 1). Omeprazole reduced the distance migrated when both FMLP as well as ZAS were used as chemoattractants. Incuba-

TABLE II Distribution of marker proteins in fractions from Percoll gradients. Values given are percentages of total recovery from the gradients. (Values median (range) of four experiments)

	Fraction		
	A	B	C
Alkaline phosphatase	96 (89–100)	4 (0–11)	0
Lysozyme	2 (0–3)	55 (43–76)	39 (22–49)
β-glucuronidase	6 (0–11)	24 (11–35)	70 (59–81)
Myeloperoxidase	1 (0–4)	31 (25–50)	58 (48–68)

Figure 2: Production of O₂⁻ by polymorphonuclear neutrophils preincubated with omeprazole in varying concentrations and activated with FMLP 10⁻⁷ mol/l). (Values mean (SD).)

tion with omeprazole (5 × 10⁻⁵ and 10⁻⁴ mol/l; 30 minutes) and resuspension in media without omeprazole reduced the chemotactic index to 61–65% and 53–63% of untreated control cells (n=3). When acid degraded omeprazole was used a similar inhibition was observed (Table I).

GENERATION OF O₂⁻

PMN generation of O₂⁻ was inhibited by omeprazole in a dose dependent manner (Fig 2). There was 50% inhibition (IC₅₀) at a concentration of 2.5 × 10⁻⁵ mol/l.

Varying the time that the PMNs were incubated with omeprazole from 10 to 50 minutes showed the effect to be maximal after 30 minutes (10 minutes: 82%; 20 minutes: 62%; 30 minutes: 35%; 50 minutes: 34%; n=3).

Quenching of O₂⁻ generated by omeprazole would mimic inhibition of ferricytochrome c reduction. A cell free xanthine-xanthine oxidase system was used to assess the effect of omeprazole alone. In this system omeprazole did not scavenge the O₂⁻ generated (2 × 10⁻⁴–5 × 10⁻⁴

Figure 3: Release of lysozyme, β-glucuronidase, and lactate dehydrogenase (LDH) from FMLP stimulated polymorphonuclear neutrophils preincubated with omeprazole in the concentrations indicated. (Values mean (SD) of seven experiments.)

Figure 4: Distribution of cytochrome b_{-245} in membranes (fraction A) and granules (fraction B) from PMNs after incubation with omeprazole, solvent polyethylene glycol 400 (PEG400) or inactive cells (control). (Values mean (SD) of four experiments.)

mol/l; O_2^- generation: 101–106% of controls ($n=3$)).

Generation of O_2^- was also measured after incubation of PMNs with acid degraded omeprazole, imidazole, and 5,6-di-methyl-benzimidazole. Only acid degraded omeprazole (10^{-5} – 5×10^{-4} mol/l) was found to inhibit O_2^- generation by 92 to 0% of control incubations. Imidazole or 5,6-di methylbenzimidazole had no effect in concentrations from 10^{-5} to 5×10^{-4} mol/l.

DEGRANULATION OF PMNS

Preincubation with omeprazole followed by activation of PMNs showed a diminished liberation of enzymatic activity into the medium in concentrations above 10^{-4} mol/l. The marker for cell damage (lactate dehydrogenase) remained low (control: 6% (range 5–8); omeprazole 5×10^{-4} mol/l: 5% (range 0–12) (Fig 3).

TRANSLOCATION OF CYTOCHROME b_{-245}

Nitrogen cavitation of PMNs and centrifugation on Percoll gradients results in three distinct bands. Table II gives the distribution of marker proteins. Fraction A contains the marker for membranes (alkaline phosphatase) and fractions B and C markers for granules (β -glucuronidase, lysozyme, myeloperoxidase).

Spectroscopy of reduced *versus* non-reduced samples showed translocation of cytochrome b_{-245} from the granule fraction to the membrane fraction after activation. Figure 4 depicts the distribution of cytochrome b in resting, activated, and activated omeprazole treated PMNs. Incubation with omeprazole or polyethylene glycol 400 did not change the distribution of cytochrome b_{-245} .

Discussion

The present study found that omeprazole inhibits PMN chemotaxis, degranulation, and O_2^- generation in concentrations that can be

observed after intravenous administration of omeprazole.²⁴ Similar studies have not been reported before. Omeprazole inhibited the response of PMNs in a concentration dependent fashion, with an IC_{50} of 2.5×10^{-5} mol/l. This is higher than the IC_{50} for the parietal cells (IC_{50} : 5×10^{-6} mol/l). The differences in the IC_{50} required to affect PMNs and the parietal cells make a clinical effect unlikely during peroral administration since the plasma concentrations are low. However, during intravenous administration concentrations of $1\text{--}2 \times 10^{-5}$ mol/l are observed for a period of several hours.²⁴ An effect *in vivo* is not unlikely considering the irreversibility of the *in vitro* effect observed in the present study.

Omeprazole is a substituted benzimidazole with a high affinity for intracellular compartments with an acid pH.⁴ In the acid environment the drug degrades and the activity is associated with the degradation products.^{4,25} The mode of action is inhibition of the parietal cell H^+ , K^+ -ATPase, causing a diminished proton secretion into the secretory canaliculus.^{3,4}

The reasons for the present study were: (1) the affinity of omeprazole for acid environment and (2) the acidification of the phagolysosome by a proton secreting mechanism in the activated PMN.^{6,8,12} The mechanism of inhibition observed is probably an effect upon the PMN itself, since no effect could be demonstrated in a cell free system.

Increased oxidative metabolism and generation of O_2^- occur as early events in the activation of PMN. Omeprazole and its acid degradation products had a similar effect and it is probably the degradation products that are active. The acid degradation used in the present study results in generation of sulphanemid acid derivatives known to be active in the parietal cell.²⁰ Inhibition by omeprazole and its degradation products and lack of effect in a cell free system or of related imidazoles indicate that a cellular mechanism is inhibited. The findings that a period of 30 minutes is required for maximal inhibition and that the effect is irreversible point to a cellular mechanism. Several mechanisms are possible.

Omeprazole has a high affinity for the glutathione-system²⁵ and inhibition of the glutathione-system diminishes production of O_2^- by PMNs. Inhibition of the NADPH-oxidase or blocking the formation of the oxidase in the membrane itself may also have reduced O_2^- generation.

Activation of PMNs is associated with formation of a dormant membrane associated a NADPH-oxidase by translocation of cytochrome b_{-245} from the granules to the membrane.¹⁹ This translocation was not affected by omeprazole. A direct effect on the oxidase itself or on assembly of the oxidase in the membrane cannot be excluded. Imidazole in a high concentration (30–50 mmol/l) inhibits the oxidase²⁶ but imidazole and 5,6-dimethyl-benzimidazole in low concentrations did not inhibit O_2^- generation.

Omeprazole may also inhibit ATPase in the PMN membrane. A quabain sensitive ATPase has been described for the PMN membrane by

some²⁷ but the existence is disputed.¹⁵ During activation of PMNs an electroneutral proton secretion is activated. This includes an Na⁺H⁺ ATPase and there is evidence that this proton pump requires assembly of several components at the membrane.^{6,7,12} The present study raises the possibility of common features between the proton pump in the parietal cell and in PMNs. It is noteworthy in this context that omeprazole inhibits another phagocyte – viz the osteoclast in vitro at an IC₅₀ of 10⁻⁵ mol/l.²⁸

In conclusion, a new anti-ulcer drug, omeprazole, has been shown to inhibit the functions of PMNs in concentrations that are reached during intravenous administration. Whether these findings have clinical importance remains to be studied in vivo. A beneficial implication of this study is that omeprazole may have an anti-inflammatory activity.

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