Introduction

Although they are primarily antimicrobial, macrolide antibotics have been reported to possess anti-inflammatory properties which may explain their usefulness in the treatment of a variety of acute and chronic inflammatory disorders, including bronchial asthma, sub-acute sclerosing panbronchiolitis and ischaemic heart disease, including unstable angina and acute myocardial infarction.1 Macrolides have been reported to suppress the pro-inflammatory activities of neutrophils,2 eosinophils,3 monocytes,4 T-lymphocytes5 and bronchial epithelial cells.6 The anti-inflammatory activities of macrolides are well documented, but the molecular and biochemical basis of these potentially beneficial, albeit secondary, properties is not completely understood. However, some insights may be provided by the finding that only 14- and 15-member macrolides, and not 16-member agents such as spiramycin and josamycin, possess significant anti-inflammatory activity.1,3 We have reported previously that the anti-inflammatory and cytoprotective properties of 14-member (clarithromycin, erythromycin and roxithromycin) and 15-member (azithromycin) macrolides are closely correlated with their membrane-stabilizing activity.2 However, the membrane-stabilizing and anti-inflammatory potential of 16-member macrolides was not investigated. In the current study we have compared the effects of spiramycin with those of clarithromycin on the generation of superoxide by stimulated human neutrophils in vitro in relation to the membrane-stabilizing activities of these agents.

Materials and methods

Macrolides

Clarithromycin and spiramycin were kindly provided by the Johannesburg-based South African affiliates of Abbott Laboratories (Abbott Park, IL, USA) and Rhône–Poulenc Rorer (Vitry-sur-Seine, France), respectively. Unless stated, all other agents used were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Neutrophils

Human neutrophils were obtained from heparinized (5 units of preservative-free heparin/mL) venous blood of healthy adult volunteers and separated from mononuclear cells by gradient centrifugation. Human neutrophils were activated by incubation with 1 µM N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). The effects of the 16-member macrolide spiramycin (2.5–80 mg/L) and the 14-member agent clarithromycin on the production of superoxide by activated human neutrophils were compared in vitro and related to membrane-stabilizing activity. Superoxide production was measured by lucigenin-enhanced chemiluminescence with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (1 µM) as the stimulus, and membrane-stabilizing activity was measured by a haemolytic procedure. Clarithromycin, but not spiramycin, caused dose-related inhibition of superoxide production by activated neutrophils and also protected erythrocytes against haemolysis, while spiramycin possessed only weak membrane-stabilizing activity. These observations underscore the apparent association between the anti-inflammatory and membrane-stabilizing properties of macrolides.

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leucocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS; 0.15 M, pH 7.4) and sedimented with 3% gelatin for 15 min at 37°C to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity and viability, were resuspended to 1 × 10⁶ per mL in PBS and held on ice until used.

**Superoxide generation**

Superoxide generation was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method. Neutrophils were pre-incubated for 15 min at room temperature in 900 μL Hanks’ balanced salt solution (HBSS) containing 0.2 mM lucigenin in the presence or absence of the macrolides (1.25–80 mg/L) followed by 15 min at 37°C. Spontaneous and stimulus-activated LECL responses were then recorded in an LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of 100 μL of N-formyl-L-methionyl-L-leucyl-phenylalanine (FMLP; 1 μM) which was used as the stimulus of membrane-associated oxidative metabolism. LECL readings were integrated for 5 s intervals and recorded as mV/s.

**Membrane stabilization**

The membrane-stabilizing potential of spiramycin and clarithromycin was measured using a haemolytic assay. Sheep erythrocytes were washed three times and resuspended to 0.5% in HBSS. The erythrocytes (final concentration 0.05%) were then co-incubated with the macrolides (40 or 80 mg/L) for 30 min at 37°C followed by addition of the membrane-destabilizing lysophospholipid, lysophosphatidylcholine (LPC) at concentrations (0.6–0.75 mg/L) that caused partial haemolysis. After 5 min the erythrocytes were pelleted by centrifugation and the supernatants were assayed spectrophotometrically at 405 nm for haemoglobin content.

**Statistical analysis**

The results of each series of experiments are expressed as mean ± S.E.M. Where appropriate, levels of statistical significance were calculated by Student’s t-test.

**Results**

**Superoxide production**

The effects of the test macrolides on superoxide production by FMLP-activated neutrophils are shown in Figure 1. Clarithromycin caused dose-related inhibition of superoxide production which was statistically significant (P < 0.05) at all concentrations tested, while spiramycin, at concentrations of 2.5–20 mg/L potentiated the generation of this reactive oxidant by the cells, with very slight inhibition observed at the highest concentration tested (80 mg/L).

**Membrane stabilization**

The effects of the test macrolides on the resistance of sheep erythrocytes to lysis by LPC are shown in Figure 2. Clarithromycin, at both concentrations tested (40 and 80 mg/L) caused dose-related inhibition of haemolysis, while spiramycin caused a slight increase in haemolysis at the highest concentration tested (80 mg/L).
Macrolides and neutrophils

80 mg/L), caused significant ($P < 0.05$) protection against LPC-mediated haemolysis, while spiramycin had only modest membrane-stabilizing activity, which was statistically significant ($P < 0.05$) at the higher concentration tested.

Discussion

The inhibitory effects of 14- and 15-member macrolides and ketolides on the production of pro-inflammatory, toxic reactive oxidants by neutrophils are well established and have been attributed to the membrane-stabilizing properties of these agents and to their inhibitory effects on the phospholipase D/phosphatidic acid phosphohydrolase pathway. Since this latter pathway is prone to inhibition by membrane-stabilizing agents, these two mechanisms are not mutually exclusive. The inhibitory effects of macrolides and ketolides on superoxide production by activated neutrophils, which are related to membrane-stabilizing mechanisms, may be achieved by interference with the protein motions, both lateral and rotational, which are required for optimum functioning of NADPH oxidase, the superoxide-generating system of phagocytes. Interference with phospholipase D/phosphatidic acid phosphohydrolase may also contribute to the anti-oxidative interactions of these agents with neutrophils by decreasing the production of second messengers involved in activation of the latent oxidase.

In the current study we investigated the anti-oxidative and membrane-stabilizing potential of the 16-member macrolide spiramycin. Unlike clarithromycin, which was effective at therapeutically relevant concentrations, this agent had no significant inhibitory effects on superoxide production by activated neutrophils. Indeed, production of this reactive oxidant was in fact increased in the presence of spiramycin at concentrations of 2.5–20 mg/L, with only slight inhibition observed at the highest concentration tested. These findings are in agreement with two previous studies which reported either no effects or slight stimulation of FMLP-activated superoxide production by neutrophils co-incubated with spiramycin at the concentration range used in the present study.

Interestingly, the inability of spiramycin to inhibit the oxidative burst in stimulated neutrophils was associated with weak membrane-stabilizing activity in comparison with that of clarithromycin. The reason for this remains to be established, but may result from structural differences between these agents and/or lower uptake of spiramycin by eukaryotic cells relative to that of clarithromycin. The detection of membrane stabilization, in which cytolysis is the end-point, necessitated the use of high concentrations of clarithromycin and does not exclude more subtle membrane-stabilizing actions such as inhibition of superoxide production at lower concentrations of this macrolide. The physicochemical and biochemical mechanism by which 14- and 15-member macrolides promote membrane stabilization remains to be established, but does not appear to be achieved by direct inhibition of phospholipase A2 or by neutralizing interactions with bioactive phospholipids.

In conclusion, the 16-member macrolide spiramycin had no meaningful inhibitory action on superoxide production by activated neutrophils in vitro, which contrasts with the effects of clarithromycin. Spiramycin's relative lack of anti-inflammatory properties appears to correlate with the relatively weak membrane-stabilizing activity of this agent.

References


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