Erythromycin Does Not Directly Affect Neutrophil Functions

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To determine whether erythromycin could affect neutrophil functions, we measured N-formyl-methionyl-leucyl-phenylalanine (FMLP)-induced chemotaxis and superoxide generation of neutrophils in the presence of erythromycin at various concentrations. Erythromycin had no effect on either of them. We further confirmed that intracellular free calcium concentration ([Ca²⁺]i) was not influenced by FMLP stimulation in the presence of erythromycin. Our results indicate that erythromycin has no direct effects on neutrophil functions in vitro, although it is reported that erythromycin inhibits the local migration of neutrophils in the small airways of subjects with asthma. (Chest 1994; 105: 520-23)

Current studies emphasize that bronchial hyperresponsiveness is a characteristic feature of bronchial asthma, and airway inflammation plays an important role in bronchial hyperresponsiveness. Increased neutrophils are found in the airways of patients with asthma after epithelial injury on the airway.

Several investigators showed that erythromycin itself had anti-inflammatory actions, such as the inhibition of chemotaxis, and the generation of reactive oxygen species by neutrophils. On the other hand, Anderson reported that erythromycin potentiated the human neutrophil chemotaxis. It remains controversial whether erythromycin may alter neutrophil functions.

To clarify the nonantibiotic effects of erythromycin, we investigated the effects of erythromycin on neutrophil chemotaxis and superoxide (O₂⁻) generation. To further understand its nonantibiotic properties, we measured changes in intracellular free calcium concentration ([Ca²⁺]i), which is considered to be an essential event for cell activation.

Materials and Methods

Reagents

Erythromycin was provided by Shionogi Pharmaceutical Co (Osaka, Japan). The N-formyl-methionyl-leucyl-phenylalanine (FMLP), cytochrome c, and superoxide dismutase were purchased from Sigma Chemical Co (St Louis, Mo). Fura-2-acetoxymethyl ester (fura-2/AM) was obtained from Dojin Laboratory (Kumamoto, Japan). Ethylene-glycol-O,O'-bis(2-aminoethyl)-N,N',N'-tetra-acetic acid (EGTA) was from Wako Pure Chemicals (Osaka, Japan). Other reagents of analytical grade were purchased from commercial sources. Erythromycin was dissolved in ethanol. The final concentration of ethanol was below 0.5 percent.

Preparation of Neutrophils

Neutrophils were separated from the blood of healthy volunteers as previously described. After the dextran sedimentation of the whole blood, erythrocytes were removed by hypotonic lysis. Neutrophils were obtained by Ficoll-Hypaque gradient centrifugation and washed twice with phosphate-buffered saline solution (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH₂PO₄, and 1.5 mM K₂HPO₄; pH 7.4).

Chemotaxis Assay

Chemotaxis assays were performed on a 96-well microchemotaxis chamber (Neuro Probe Inc, Bethesda, Md). To the bottom wells, 25 μl of the chemotaxant (FMLP) or the N-2-hydroxyethylpyperazine-N'-2-ethanesulfonic acid (HEPES) solution (20 mM HEPES, 135 mM NaCl, 5 mM KCl and 5 mM D-glucose) containing 0.1 percent bovine serum albumin were added. A polycarbonate filter sheet, without polyvinylpyrrolidone, containing 3-μm pores was placed on the top of the wells in the bottom plate. A gasket and top plate were fixed in place. The neutrophils were suspended at a concentration of 1 × 10⁶ cells/ml in the HEPES solution, 50 mM of the control or erythromycin-treated neutrophils were added to each of the top wells. The entire assembly was incubated for 60 min at 37°C in humidified air with 5 percent CO₂. After incubation, the filter was fixed in methanol. The filter was air-dried and stained with Diff-Quik solutions (Kokusai Chemicals, Kobe, Japan). After nonsmigreated cells were washed, the stained filter was analyzed by a microplate reader (BIO-RAD Laboratory, Richmond, Calif) as an absorbance at 595 nm.

Assay of O₂⁻ Generation

The O₂⁻ generation of FMLP-stimulated neutrophils was measured by the determination of superoxide dismutase-inhibitable reduction of cytochrome c. Reaction mixtures (1 ml), which consisted of PBS (pH 7.4) containing 10⁶ neutrophils, 50 mM cytochrome c, 0.5 mM CaCl₂, and 0.5 mM MgSO₄, were incubated for 10 min or 2 h at 37°C with or without various concentrations of erythromycin. After addition of FMLP (final concentration of 1 μM), the absorbance change (550 to 540 nm) was followed on the dual-wavelength spectrophotometer (Shimadzu UV-160 A, Japan) and was converted to the O₂⁻ release with a molar absorption coefficient of reduced minus oxidized cytochrome c as 19.1·10⁶ mmol/L·cm⁻¹.

Measurement of [Ca²⁺]i

Neutrophils (2 × 10⁵ cells/ml) were incubated for 30 min at 30°C in the HEPES solution containing 2 μmol fura-2/AM. The loaded cells were washed and resuspended in the HEPES solution to a density of 2 × 10⁶ cells/ml. Suspensions (2.5 ml) of fura-2-loaded neutrophils containing 1 mM CaCl₂ were incubated with or without erythromycin for 10 min. After stimulation by 1 μmol FMLP, the fluorescence was

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measured with a spectrophotometer (Shimadzu RF-5000, Japan) at excitation wavelengths of 340 and 390 nm, and an emission wavelength of 490 nm. Following the measurements, the cells were lysed by adding 25 μl of 5 percent polyoxyethylene(10)p-tert-octylphenol (Triton X-100) for the determination of Fmax. Fmin was then determined by adding 25 μl of 1 mol EGTA to the neutrophil lysate. The [Ca2+]i was calculated by a previously published method11 with a distribution coefficient value of 224 nmol for Ca2+.

Statistical Analysis

Data are reported as mean ± standard error (SE) of the mean. Statistical comparisons were made with Student's t test. Significance was accepted with a p value of less than 0.05.

RESULTS

Chemotaxis

The effect of erythromycin on the mobility of neutrophils was studied with FMLP. The optimal concentration of FMLP (10−7 mol/L) was determined by a dose-response curve, as shown in Figure 1. Neutrophils were incubated with or without various concentrations of erythromycin during the chemotaxis assay. The effects of erythromycin on the mobility of neutrophils are shown in Figure 2, where the activities are expressed as a percentage of the control activity obtained from the stimulated neutrophils in the absence of erythromycin. At any of the concentrations (1 to 100 μg/ml), erythromycin did not alter the direct movement of the neutrophils. We confirmed that results obtained by our method, using microplate reader, correlated well with those of the conventional method, counting migrated neutrophils under microscope.

In other series of experiments, we treated neutrophils with or without erythromycin in the assay buffer at 37°C for 2 h before applying cells to the chemotactic chamber. The mobility of neutrophils remarkably decreased in all groups including the control (no erythromycin treated group).

O2− Generation

Neutrophils generated O2− by addition of 1 μmol FMLP at the maximal rate of 2.67 ± 0.13 nmol/min/106 cells. After preincubation with various concentrations of erythromycin for 10 min at 37°C, the reaction was initiated by addition of FMLP. The effects of erythromycin on the O2− generation are shown in Figure 3. The FMLP-induced neutrophil O2− generation was not affected by erythromycin at any of the concentrations (1 to 100 mg/ml). Activity of O2− generation of neutrophils was maintained at the initial level until 180 min incubation with erythromycin in the assay buffer at 37°C, started decreasing after 210 min down to undetectable level after 14 h (data not shown). We, therefore, examined O2− genera-
Concentration (μg/ml) of [Ca²⁺]i in neutrophils stimulated by FMLP. Results are expressed as the mean ± SE of three separate experiments. (□): resting level of [Ca²⁺]i; (■): peak level of [Ca²⁺]i; stimulated by 1 μM FMLP. None of the differences were found statistically significant at any of the concentrations.

Chemotaxis and O₂⁻ generation of neutrophils are preceded by an increase in [Ca²⁺]i. Stimulation of neutrophils by 1 μM FMLP raised [Ca²⁺]i from a resting concentration of 69.1 ± 5.9 nmol to 278.1 ± 11.9 nmol within several seconds. Incubation with erythromycin (1 to 100 μg/ml) affected neither the resting concentration nor the FMLP-induced increase of [Ca²⁺]i (Fig 4).

**DISCUSSION**

Erythromycin is one of the antibiotics extensively used worldwide. It is reported to have anti-inflammatory actions, such as inhibition of neutrophil chemotaxis and inhibition of O₂⁻ generation. Erythromycin has been reported to be effective in adult patients with diffuse panbronchiolitis. Mikami reported that the mechanism of its action is mainly a result of the inhibitory effect on neutrophil chemotaxis. On the other hand, Anderson reported that erythromycin potentiated neutrophil chemotaxis, and Eyraud et al reported that it did not affect neutrophil migration. Whether anti-inflammatory effects of erythromycin are caused by activating human neutrophil functions remains controversial. In the present study, it was observed that neither chemotaxis nor O₂⁻ generation of neutrophils was affected by short-time exposure (10 min) to erythromycin. Longer exposure to erythromycin (2 h) did not alter O₂⁻ generation either. We, however, could not confirm whether long-time exposure to erythromycin affected chemotactic activity, because 2h preincubation of cells with erythromycin in the assay buffer at 37°C before applying chemotactic chamber almost completely diminished mobility of cells in all groups including the control.

Erythromycin had no influence on the increase of [Ca²⁺]i, which is essential for the neutrophil activation induced by FMLP. Binding of FMLP to its receptors first causes an increase of [Ca²⁺]i and then activates protein kinase C, which in turn leads to the activation of chemotaxis and reduced nicotinamide adenine dinucleotide phosphate oxidase, the enzyme response O₂⁻ generation. The increase [Ca²⁺]i is known to proceed in two steps: an initial rise occurs within 10 ~ 15 s, and a sustained second rise within 0.5 ~ 2 min. The initial rise is due to the release of intracellular Ca²⁺ from its store site, the second rise resulting from the influx of extracellular Ca²⁺. In addition, we confirmed that in neutrophils some anti-allergic drugs, such as azelastine, tranilast, and pemirolast, remarkably inhibited O₂⁻ generation accompanied by the inhibition of the increase of [Ca²⁺]i.

In this present study, erythromycin affected neither the initial rise nor the second rise (data not shown). Therefore, it is unlikely that erythromycin could directly alter neutrophil functions without changing [Ca²⁺]i.

The present study does not necessarily rule out the possibility that erythromycin may reduce bronchial hyperresponsiveness through the action of neutrophils. It is clinically observed that erythromycin does inhibit the local migration of neutrophils in the airways of patients with panbronchiolitis. Recently, Goswami et al reported that erythromycin inhibited respiratory glycoconjugate secretion, suggesting that erythromycin affects mucus clearance. Further studies including long-time effects of EM with more adequate experimental design are necessary to evaluate possible beneficial nonantibiotic effects of erythromycin.

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