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# Human and rat alveolar macrophages express multiple EDG receptors

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**Abstract**

Endothelial differentiation gene (EDG) receptors are a new family of eight G protein-coupled receptors for the lysophospholipids lysophosphatitic acid and sphingosine-1-phosphate. In the present experiments, the expression of EDG receptors in rat and human alveolar macrophages was studied by reverse transcription-polymerase chain reaction (RT-PCR). In alveolar macrophages of both species, mRNA for multiple EDG receptors could be detected, but the pattern of expression was different in both species. In human alveolar macrophages, mRNA for EDG1, EDG2, EDG4, EDG7 receptors and, to a lesser extent, for the EDG7 receptor was detected, whereas in rat macrophages, mRNA for EDG2, EDG5 receptors and, to a lesser extent, for the EDG6 receptor was found. In functional experiments, it was observed that lysophosphatitic acid and sphingosine-1-phosphate can stimulate $O_2^-$ generation in rat and human alveolar macrophages suggesting that lysophosphatitic acid and sphingosine-1-phosphate possibly acting via EDG receptors may play a role in controlling the activation of macrophages. 

*Keywords:* Alveolar macrophage; Lysophospholipid; EDG receptor; Lysophosphatitic acid; Sphingosine-1-phosphate; Oxygen radical

## 1. Introduction

Lipid mediators derived from membrane glycerophospholipids and sphingolipids have first been characterized as important intracellular messenger (for review, see Meyer zu Heringdorf et al., 1997; Spiegel and Merrill, 1996; Hla et al., 1999), but recently, it was discovered that lysophosphatitic acid and sphingosine-1-phosphate are also potent agonists for a rapidly growing family of G protein-coupled receptors (for review, see An et al., 1998; Hla et al., 1999; Racké et al., 2000). The first member of this family had been described as an immediated-early gene product expressed in phorbol ester activated endothelial cells. Since it showed significant sequence homology to the G protein-coupled receptor superfamily, it was considered as an orphan G protein-coupled receptor (Hla and Maciag, 1990) and named endothelial differentiation gene (EDG) receptor. In the meantime, eight different EDG receptors have been cloned and characterized in different expression systems. They are activated by lysophosphatitic acid and sphingosine-1-phosphate, but EDG1, EDG3, EDG5, EDG6 and EDG8 receptors prefer sphingosine-1-phosphate, whereas EDG2, EDG4 and EDG7 receptors prefer lysophosphatitic acid as agonist. Moreover, it has been shown that they can couple to a variety of intracellular signals (for review, see An et al., 1998; Hla et al., 1999; Racké et al., 2000). Nevertheless, the physiological functions of EDG receptors remain to be determined.

It has been shown that lysophospholipids are elevated in extracellular fluid of inflamed tissue (Murakami et al., 1997), and there is indirect evidence that this may also be true in inflammatory airway diseases. Thus, secretory phospholipase $A_2$, which can release lysophosphatitic acid from phospholipids of perturbated cell membranes (Fourcade et al., 1995), was found to be increased in broncho-alveolar lavage fluid of ovalbumin-sensitized guinea-pigs (Sane et al., 1996) and of antigen-challenged allergic asthmatics (Chilton et al., 1996; Bowton et al., 1997). Moreover, an acid sphingomyelinase, a key enzyme in the formation of sphingosine-1-phosphate, has been found to be secreted by macrophages as well as other cells (Schissel et al., 1996; Marathe et al., 1998), and this process could be stimulated by cytokines. As acid sphingomyelinase is particularly active in an acid environment, as present, for example, in inflamed tissue, a role of sphingosine-1-phosphate in inflammatory reactions appears likely.

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The aim of the present study was to test whether EDG receptors, the putative targets for lysophosphatitic acid and sphingosine-1-phosphate are expressed in rat and human alveolar macrophages, and whether lysophosphatitic acid or sphingosine-1-phosphate might cause an activation of these cells.

## 2. Materials and methods

### 2.1. Materials

Amphotericin B, Dulbecco's modification of Eagle's/Ham's F-12 medium (DME/F-12 medium), formylmethionyl-leucyl-phenylalanine (fMLP), *p*-iodonitrotetrazolium violet, lipopolysaccharides from *Escherichia coli* 0127:B8, lysophosphatitic acid; phorbol myristate acetate (PMA), RedTaq DNA polymerase, sphingosine-1-phosphate, penicillin-streptomycin solution, (all Sigma, München, Germany); fetal calf serum (Vitromex, Germany); Trizol® reagent for RNA isolation (Life Technologies, Karlsruhe, Germany); avian myoblastosis virus (AMV) reverse transcriptase (Promega, Mannheim, Germany). All deoxynucleotides for RT-PCR were obtained from MWG Biotech (Ebersberg, Germany).

### 2.2. Preparation and culture of alveolar macrophages

#### 2.2.1. Rat alveolar macrophages

Sprague Dawley rats (own breeding) of either sex were killed by stunning followed by exsanguination. Lung and trachea were excised en bloc and lavaged thrice by instilling 10–15 ml of cold phosphate-buffered saline (see Hey et al., 1995). Usually, for one preparation of alveolar macrophages, lavage fluids from 4 to 6 lungs were pooled and centrifuged at $400 \times g$ for 10 min. Thereafter, cells were resuspended in DME/F-12 medium supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml amphotericin B, and were disseminated, $4 \times 10^6$ alveolar macrophages on 35-mm culture dishes (for RNA preparation) or $10^6$ alveolar macrophages/well on 24-well plates (for determination of respiratory burst). Alveolar macrophages were allowed to adhere for 2 h (37 °C; 5% $CO_2$), before the medium was renewed to remove non-adherent cells. The adherent cells consisted of more than 95% alveolar macrophages according to morphological criteria (May Grünwald-Giemsa staining as described by Rick, 1974). Thereafter, alveolar macrophages were cultured for up to 20 h.

#### 2.2.2. Human alveolar macrophages

Cells were obtained from patients by broncho-alveolar lavage, which was performed for diagnostic purpose. The protocol for obtaining the samples was approved by the ethics review board for human studies (Medizinische Einrichtungen der Rheinische Friedrich-Wilhelms-Universität Bonn), and patients gave informed consent. Cells obtained by broncho-alveolar lavage were washed, disseminated and cultured as described above for rat alveolar macrophages.

### 2.3. Extraction of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared and the first strand cDNA synthesized as described previously (Hammermann et al., 1998; Racké et al., 1998). Oligonucleotide primers were constructed based on EMBL sequences: rat β-actin, 5′-TTCTACAATGAGCTGCGTGTGGC-3′ and 5′-AGAGGTCTTTACGGATGTCAACG-3′; rat EDG-1, 5′-CTTCAGCCTCCTTGCTATCG-3′ and 5′-GCAGGCAATGAAGACGACACTCA-3′; rat EDG-2, 5′-ATTTCACAGCCCCAGTTCAC-3′ and 5′-ACAATAAAGGCACCCAGCAC-3′; rat EDG-3, 5′-TCAGGGAGGGCAGTATGTTC-3′ and 5′-CTGACTCTTGAAGAGGATGG-3′; mouse/human EDG-4, 5′-ACTACAACGAGACCATCGGC and 5′-AAGGGTGGAGTCCATCAGTG; rat EDG-5, 5′-TTCTGGTGCTAATCGCAGTG-3′ and 5′-GAGCAGAGAGTTGAGGGTGG-3′; rat EDG-6, 5′-GTGCTCAACTCAGCCATCAA and 5′-CTGCCAAACATTCATCATGG; rat EDG-7, 5′-TGAGCCTCCATGTGTAGCTG and 5′-AGCTTGTGCAGCCTCTCTTC; rat EDG-8, 5′-TGTTCCTGCTCCTGGGTAGT-3′ and 5′-GTTTCGGTTGGTGAAGGTGT-3′; human β-actin, 5′-TTCTACAATGAGCTGCGTGTGGC-3′ and 5′-CCTGCTTGCTGATCCACATCTGC-3′; human EDG-1, 5′-CCACAACGGGAGCAATAACT-3′ and 5′-GTAAATGATGGGGTTGGTGC-3′; human EDG-2, 5′-ATTTCACAGCCCCAGTTCAC-3′ and 5′-CTGTAGAGGGGTGCCATGTT-3′; human EDG-3, 5′-TCAGGGAGGGCAGTATGTTC-3′ and 5′-CTGAGCCTTGAAGAGGATGG-3′; human EDG-4, 5′-CCATCTACTACCTGCTCGGC-3′ and 5′-AAGGGTGGAGTCATCAGTGG-3′; human EDG-5, 5′-CCAATACCTTGCTCTCTGGC-3′ and 5′-CAGAAGGAGGATGCTGAAGG-3′; human EDG-6, 5′-CGGCTCATTGTTCTGCACTA-3′ and 5′-GATCATCAGCACCGTCTTCA-3′; human EDG-7, 5′-TTAGCTGCTGCCGATTTCTT-3′ and 5′-ATGATGAGGAAGGCCATGAG-3′; human EDG-8, 5′-CAAGGCCTACGTGCTCTTCT. PCR amplification was performed using RedTaq DNA polymerase and specific primers in a programmable thermal reactor (RoboCycler®, Stratagene, Amsterdam, Europe) with initial heating for 3 min at 94 °C, followed by 35 cycles of 45-s denaturation at 94 °C, annealing at 48–61 °C (30 s), extension at 72 °C (1 min), and a final extension for 10 min at 72 °C. PCR products were separated by a 1.2% agarose gel electrophoresis and documented by a video documentation system, and the optical density of the bands was quantified by the RFLP-scan 2.01 software (MWG).

### 2.4. Measurement of superoxide generation

Superoxide generation by rat and human alveolar macrophages was measured by determination of the reduc-

tion of iodonitrotetrazolium violet to iodonitrotetrazolium formazan. Briefly, alveolar macrophages ($10^6$ cells/well) were cultured for 20 h. Thereafter, the medium was replaced by medium (0.5 ml/well) containing iodonitrotetrazolium violet (0.5 mg/ml) alone or together with test substances. After 1-h incubation at 37 °C, 0.5 ml $HCl_{conc}$ containing 5% dimethyl sulfoxide (DMSO) (v/v) were added in order to lyse the cells and dissolve iodonitrotetrazolium formazan. The cell lysate was transferred into microcuvette and the absorbance of the reduced dye determined at 492 nm.

### 2.5. Calculations and statistical analysis

Mean values of $n$ observations are given ± S.E.M. The statistical significance of differences was evaluated by analysis of variance (ANOVA) followed by the modified $t$-test of Dunnett using the computer program GraphPad InStat®. $P < 0.05$ was accepted as being significant.

## 3. Results

### 3.1. Expression of mRNA for EDG receptors

In human alveolar macrophages, mRNA for several EDG receptors was detected. As shown in Fig. 1, clear mRNA bands were detected for EDG1, EDG2, EDG4 and EDG7 receptors, but only a weak signal was detected for the EDG3 receptor. In each case, only a single amplification product of the expected size was obtained. mRNA for EDG5, EDG6 and EDG8 receptors could not be detected (data not shown). The same expression pattern of EDG receptors as observed in freshly isolated alveolar macrophages (RNA preparation after the 2 h adherence period) was found in alveolar macrophages cultured for 20 h (data not shown).

Likewise, mRNA for several EDG receptors was also detected in rat alveolar macrophages. As shown in Fig. 2, mRNA for EDG2 and EDG5 receptors was clearly detected in rat alveolar macrophages. mRNA for EDG6 was only weakly expressed in rat alveolar macrophages, but a marked signal was found in rat lung tissue. As of present, the sequences for the EDG4 receptor is only known for mouse and human, but not yet for rat; primers for the detection of mRNA of this receptor in rat alveolar macrophages were designed to meet 100% homology between the rat and human cDNA. Using such a primer pair, mRNA for EDG4 receptors could be detected in rat lung tissue, but only a very weak signal (seen at the original gel, but not in Fig. 2) was detected in rat alveolar macrophages. mRNA for EDG1 and EDG3 receptors could not be detected in rat alveolar macrophages, although mRNA for these receptors appears to be clearly expressed in the rat lung tissue (Fig. 2). Finally, mRNA for EDG7 and EDG8 receptors was not detectable in rat alveolar macrophages nor in lung tissue (data not shown).

### 3.2. Effect of lysophosphatitic acid and sphingosine-1-phosphate on respiratory burst

In rat alveolar macrophages, 1-h exposure to lysophosphatitic acid and sphingosine-1-phosphate caused an increase in $O_2^-$ generation by 80% and 150%, respectively, as detected by measuring the formation of iodonitrotetrazolium formazan from iodonitrotetrazolium violet. The magnitude of this effect was comparable to the increase produced by exposure to lipopolysaccharides or fMLP,

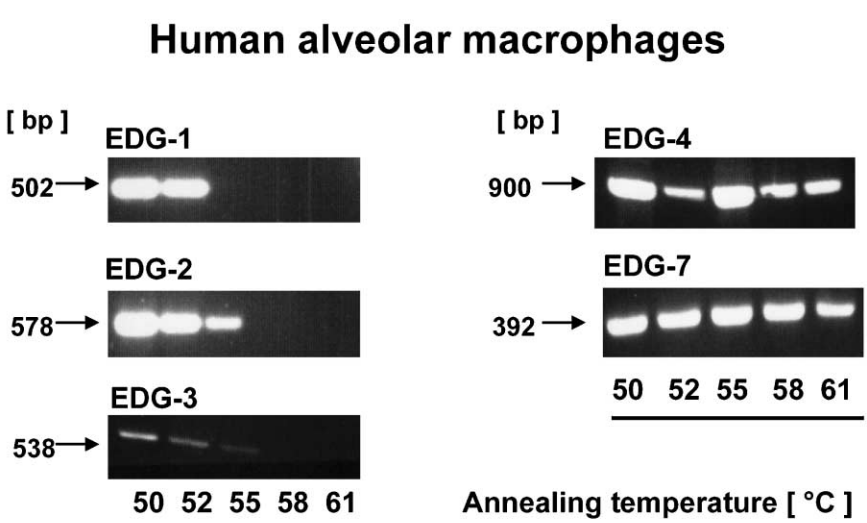


Fig. 1. Detection of mRNA for EDG receptors in human alveolar macrophages. Cells obtained by broncho-alveolar lavage were resuspended in culture medium and disseminated in 35-mm culture dishes ($4 \times 10^6$ cells). After an adherence period of 2 h, the medium together with non-adherent cells was removed and total RNA was isolated. RT-PCR was performed using primers specific for the respective human EDG receptor. Given is one out of three similar experiments.

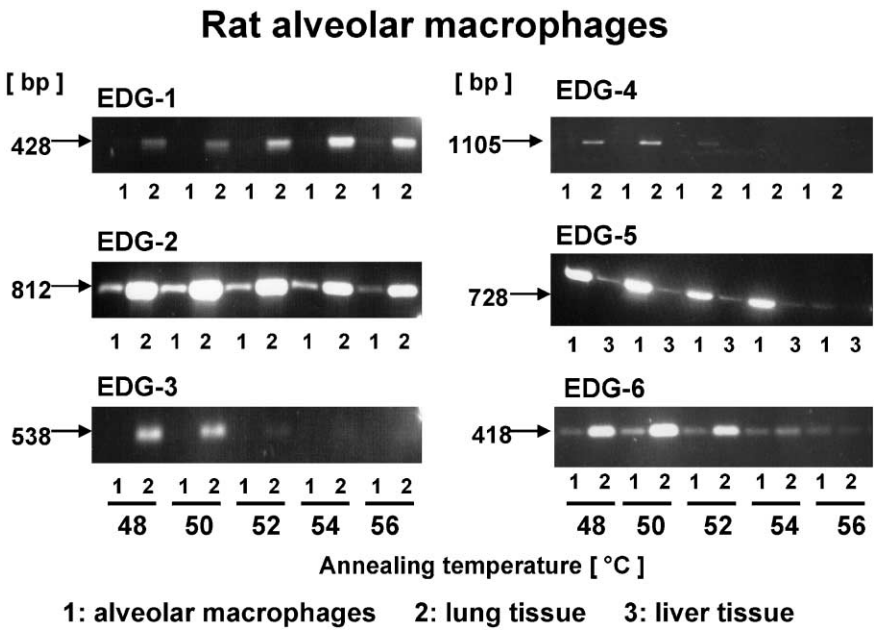


Fig. 2. Detection of mRNA for EDG receptors in rat alveolar macrophages. Cells obtained by lavage of isolated rat lungs were resuspended in culture medium and disseminated in 35-mm culture dishes ($4 \times 10^6$ cells). After an adherence period of 2 h, the medium together with non-adherent cells was removed and total RNA was isolated. RT-PCR was performed using primers specific for the respective rat EDG receptor. Given is one out of three similar experiments.

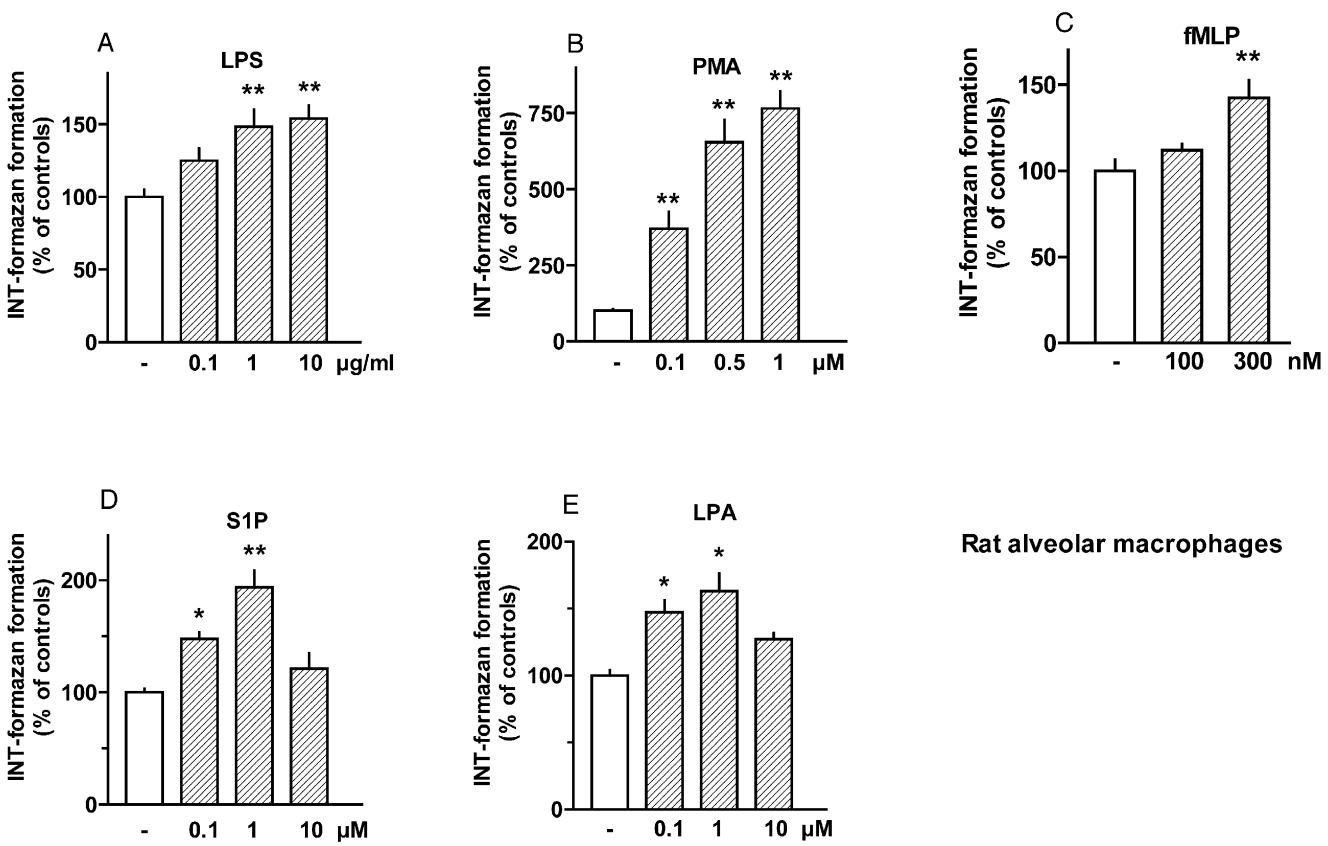


Fig. 3. Comparison of the effect of lysophosphatitic acid (LPA), sphingosine-1-phosphate (S1P), fMPL and lipopolysaccharides (LPS) on $O_2^-$ formation by rat alveolar macrophages, detected by the conversion of iodonitrotetrazolium violet to iodonitrotetrazolium (INT) formazan. Cells obtained by lavage of isolated lungs were cultured for 20 h. Thereafter, the medium was replaced by medium containing iodonitrotetrazolium violet (0.5 mg/ml) alone or in combination with the test substances at the concentrations indicated. After 1 h, the formation of iodonitrotetrazolium formazan was determined. Given are means ± S.E.M. of $n > 9$. Significance of differences from the respective controls: $^{*}P < 0.01$; $^{**}P < 0.001$.

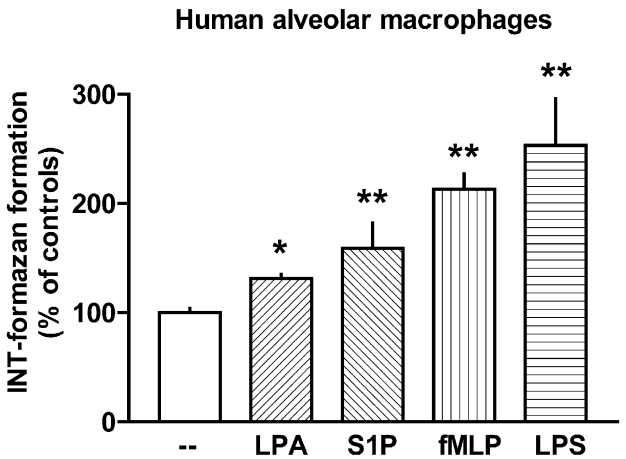


Fig. 4. Comparison of the effect of lysophosphatitic acid (LPA, 10 μM), sphingosine-1-phosphate (S1P, 1 μM), fMPL (100 nM) and lipopolysaccharides (LPS, 10 μg/ml) on $O_2^-$ formation by human alveolar macrophages, detected by the conversion of iodonitrotetrazolium violet to iodonitrotetrazolium (INT) formazan. Alveolar macrophages obtained by broncho-alveolar lavage were cultured for 20 h. Thereafter, the medium was replaced by medium containing iodonitrotetrazolium violet (0.5 mg/ml) alone or in combination with the test substances at the concentration given. After 1 h, the formation of iodonitrotetrazolium formazan was determined. Given are means ± S.E.M. of $n > 9$. Significance of differences from the respective controls: $^{*}P = 0.05$; $^{**}P < 0.01$.

which also activate $O_2^-$ generation via membrane receptors (e.g. Cohen and Glauser, 1991; Mugnai et al., 1997; Vollmar et al., 1997; Martinez and Moreno, 2000), but was substantially smaller than that evoked by the phorbol ester PMA which stimulates $O_2^-$ generation via direct activation of protein kinase C (e.g. Mugnai et al., 1997; Girón-Calle and Forman, 2000; Martinez and Moreno, 2000) (Fig. 3). In human alveolar macrophages obtained from patients without inflammatory airway diseases, 1-h exposure to lysophosphatitic acid and sphingosine-1-phosphate caused also an increase in $O_2^-$ generation by 30% and 60%, respectively (Fig. 4). This effect was, however, smaller than that evoked by fMLP or lipopolysaccharides which caused an increase by 110% and 150%, respectively (Fig. 4).

## 4. Discussion

The present study shows that different EDG receptors, the putative targets for the lysophospholipids lysophosphatitic acid and sphingosine-1-phosphate are expressed in human and rat alveolar macrophages. As already mentioned in the Introduction, lysophosphatitic acid and sphingosine-1-phosphate may act as endogenous ligands each for only a subgroup of EDG receptors, lysophosphatitic acid for EDG2, EDG4 and EDG7 receptors and sphingosine-1-phosphate for EDG1, EDG3, EDG5, EDG6 and EDG8 receptors. The present observations show that both human and rat alveolar macrophages express at least one EDG receptor for which either lysophosphatitic acid or sphingosine-1-phosphate is the preferred agonist, although the expression pattern appears to be different in both species.

Thus, in human alveolar macrophages, mRNA for several putative lysophosphatitic acid receptors, namely for EDG2, EDG4 and EDG7 receptors, was found to be expressed. In rat alveolar macrophages, EDG2 receptors, but not EDG7 receptors, appear to be expressed. The present experiments may not allow a final judgement on the expression of EDG4 receptors in rat alveolar macrophages because the PCR was performed with primers constructed from the human and mouse sequence. Although a clear signal could be obtained in rat lung tissue with these primers, they may nevertheless not meet 100% the rat sequence. Therefore, the very weak, hardly detectable signal obtained with these primers in rat alveolar macrophages may underestimate the expression of EDG4 receptor mRNA in these cells.

Of the five putative sphingosine-1-phosphate receptors, only mRNA for EDG1 and to lesser extent for EDG3 receptors was found to be expressed in human alveolar macrophages. On the other hand, in rat alveolar macrophages, only mRNA for EDG5 and lesser extent for EDG6 receptors was detected.

The expression of multiple EDG receptors in human and rat alveolar macrophages suggests that EDG receptors and lysophospholipids as their putative endogenous ligands might play a role in the control of macrophage function. Until now, however, only very little is known about functional effects of lysophospholipids on macrophages. On murine peritoneal macrophages, it was observed that lysophosphatitic acid acted as survival factor with a potency equivalent to serum (Koh et al., 1998). In the RAW 264.7, macrophages cell line lysophosphatitic acid enhanced cAMP accumulation, activated an atypical protein kinase C and caused a rise in intracellular calcium (Lin et al., 1999). In the present experiments, it was additionally shown that both lysophosphatitic acid as well as sphingosine-1-phosphate can stimulate the generation of $O_2^-$ in alveolar macrophages. The observation that lysophosphatitic acid and sphingosine-1-phosphate induced a respiratory burst comparable to the effect of lipopolysaccharides or fMLP, which also act via membrane receptors, suggests that these lysophospholipids could play a role as physiological activators of alveolar macrophages and possible of macrophages in general. Therefore, it may be speculated that lysophospholipids acting via EDG receptors might be involved in regulation of local inflammatory responses.

In conclusion, the present observations demonstrating the expression of multiple EDG receptors in rat and human alveolar macrophages should stimulate more detailed studies about effects of lysophospholipids on alveolar macrophages function. Moreover, the present results indicate that EDG receptors should be regarded as new pharmacological target by which inflammatory responses might be modulated.

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