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Differentiation by in vitro treatment of lidocaine–epinephrine and prilocaine–felypressine in neutrophils

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Abstract

Neutrophils are often the first cells of the immune system to encounter an invader, such as bacteria and fungi. Lidocaine–epinephrine induced transient potentiation of the production of superoxide anion, while prilocaine–felypressine induced persistent inhibition of the production in neutrophils. Moreover, lidocaine–epinephrine inhibited the production of hydrogen peroxide in spite that it potentiated the production of superoxide anion, while prilocaine–felypressine inhibited the production of hydrogen peroxide as well as superoxide anion. By contrast, lidocaine–epinephrine and prilocaine–felypressine are both effective in significantly inhibiting adhesion and phagocytosis. Using flow cytometric analysis, both local anesthetics were found to be effective in inhibiting the expression of Mac-1 (CD11b/CD18) in neutrophils. These results suggest that lidocaine–epinephrine and prilocaine–felypressine differentially modulate the production of superoxide anion, and could similarly inhibit adhesion, phagocytosis, and the production of hydrogen peroxide by neutrophils.

Keywords: Lidocaine–epinephrine; Prilocaine–felypressine; Neutrophil; Epinephrine; Superoxide dismutase

1. Introduction

Neutrophils are often the first cells of the immune system to encounter an invader, such as bacteria and fungi. Neutrophils are ready to leave the circulation and attack the intruder at any place at any time. The neutrophil response to infection in vivo is initiated by adherence of neutrophils to vascular endothelial cells, and progresses to the directed migration of neutrophils into the extravascular tissue space. The migration of neutrophils culminates in neutrophilmediated phagocytosis and intracellular killing of the invading microorganisms by generation of bactericidal reactive oxygen species derived from the superoxide anion radical.

Local anesthetics are widely prescribed throughout the world, and are often used during surgery where there is a very real risk of infection by pathogenic microorganisms. There is accumulating evidence that local anesthetics have immunological properties other than direct anesthetic activity. These include interactions with and alterations in functions of host phagocytes. For instance, lidocaine was found to inhibit neutrophil functions such as chemotaxis [1,2], phagocytosis [2,3], and lysosomal enzyme release and superoxide anion production [1,4]. Our earlier studies gave support to the notion that lidocaine disrupts macrophage functions [5,6]. These subjects are important, given that phagocytes are essential for controlling almost all infections and are mediators of inflammation.

These earlier findings prompted us to examine possible effects on phagocyte functions of local anesthetics. For this purpose, in this communication, we have simultaneously examined the effect of lidocaine–epinephrine and prilocaine–felypressine on functions of rat peripheral neutrophils. The studies included those on adhesion, phagocytosis, and the production of superoxide anion and hydrogen peroxide.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate (FITC)-conjugated rat monoclonal antibody against CD11b and anti-ED anti-

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body were obtained from Pharmingen (San Diego, CA). Fluorescein conjugated *Escherichia coli* (K-12) bioparticles and 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) were purchased from Molecular Probes Inc. (Eugene, OA). Lidocaine–epinephrine and prilocaine–felypressine were supplied by Astra Japan Ltd. (Osaka, Japan). Other chemicals used were all of the highest purity commercially available.

2.2. Isolation of neutrophils from peritoneum

The protocol employed here meets the guidelines of the Japanese Society for Pharmacology. All efforts were made to minimize animal suffering and to reduce the number of animals used. Neutrophils were isolated by peritoneal lavage from adult male Wistar rats weighing 200–250 g, 4 h after injection of 20 ml of 1% glycogen. Lavage was performed by washing the peritoneal cavity with 100 ml of ice-cold phosphate buffered saline (PBS, pH 7.2) supplemented with 20 U/ml heparin and 1 mM EDTA. Care was taken not to cause internal bleeding while collecting exudative neutrophils [7]. Washed neutrophils were suspended in cold Dulbecco's modified eagle medium (DMEM). Purified neutrophils were composed of a cell population $<2\%$ of which stained with anti-ED antibody (a macrophage marker).

2.3. Adhesion assay

Adhesion assays were performed by a method earlier described [8]. In brief, neutrophils at 4×10^6 were labeled by adding 15 μM CFSE for 15 min at room temperature in the dark. Neutrophils were then washed once and resuspended in PBS with 1 mM EDTA. Labeled neutrophils at 5×10^4 per well were incubated with the local anesthetics at one of three concentrations in fibronectin-coated 96-well plates for 1 h at 37°C in humidified atmosphere containing 5% CO_2 in either the presence or absence of phorbol 12-myristate 13-acetate (PMA) at 200 nM. Non-adherent cells were removed by washing, and the fluorescence intensity of the adherent neutrophils was determined at 485 nm excitation and 535 nm emission wavelengths using a multilabel counter (Wallac, Turku, Finland).

2.4. Flow cytometry assay

For staining of Mac-1 (CD11b/CD18), neutrophils adjusted to 2×10^6 cells per ml were incubated with one of two local anesthetics at one of three concentrations for 1 h in either the presence or absence of PMA at 200 nM. After one wash with PBS, neutrophils were incubated with 1 μg FITC-conjugated monoclonal antibody against CD11b or a non-specific rat antibody as a negative control for 30 min at 4°C in the dark. Neutrophils were then washed thrice with PBS, followed by suspension in 1 ml of PBS. Finally, stained cells were analyzed on a flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA) for Mac-1 expression [9]. Data are expressed as peak channel for each sample as calculated by the CellQuest® software (Becton Dickinson, Mountain View, CA).

2.5. Phagocytosis assay

Phagocytosis assay was performed by a method earlier described [10] with minor modifications [11]. In brief, neutrophils adjusted to 2×10^6/ml were adhered for 1 h. The local anesthetics at one of three concentrations and fluorescein conjugated *E. coli* (K-12) bioparticles adjusted to 1×10^7/ml were added to the adherent neutrophils. After incubation for 1 h, extracellular fluorescence was quenched by adding 25 μg trypan blue in 13 mM citrate buffer (pH 4.4). The dye was removed after 1 min and the fluorescence intensity was determined at 485 nm excitation and 535 nm emission wavelengths using a multilabel counter (Wallac, Turku, Finland).

2.6. Superoxide anion production assay

Superoxide anion production was detected by a method earlier described [12] with minor modifications [13]. Superoxide anion production was induced by stimulation of 1×10^6 neutrophils per 100 μl with 200 nM PMA. In brief, neutrophils were preincubated with the local anesthetics at one of three concentrations containing 160 μM ferricytochrome *c* for 15 min. Following the addition of PMA, incubation was initiated. Superoxide anion production was followed by cytochrome *c* reduction. Non-specific reduction of cytochrome *c* was defined by 600 U/ml superoxide dismutase. The absorbance at a wavelength of 550 nm was measured with the aid of a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at different times after incubation. Results were converted to nmol of cytochrome *c* reduced by using the extinction coefficient $E(550)$: 2.1×10^4/M/cm which means the extinction coefficient.

2.7. Hydrogen peroxide production assay

Hydrogen peroxide production was detected by a method earlier described [14] with minor modifications [11]. In brief, neutrophils (1×10^6) were preincubated with the local anesthetics at one of three concentrations containing 200 μg/ml phenol red and 17 U/ml horseradish peroxidase for 15 min. Incuba-

tion was initiated by the addition of PMA at 200 nM. Hydrogen peroxide production was followed by phenol red oxidation. Non-specific oxidation of phenol red was defined by 80 μg/ml catalase. After incubation, 10 μl of 0.5 N NaOH was added. The absorbance at a wavelength of 600 nm was measured with the aid of a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 5 min after the addition of NaOH solutions.

2.8. Data analyses

Results were all expressed as the mean ± S.E.M. and the statistical significance was determined by the two-tailed Student's *t*-test or one-way analysis of variance followed by estimation of the least significant difference.

3. Results

3.1. Adhesion

The addition of lidocaine at 1 mg/ml–epinephrine at 3.4 μM significantly inhibited the adhesion of neutrophils (Fig. 1, left panel). Similarly, prilocaine at 1 mg/ml–felypressine at 1 mU/ml was effective in reducing the adhesion of neutrophils (Fig. 1, right panel). However, no marked changes were found in the adhesion of neutrophils by the addition of these drugs at other concentrations. In contrast, PMA at 200 nM potentiated the adhesion of neutrophils (Fig. 2). In PMA-stimulated neutrophils, lidocaine–epinephrine and prilocaine–felypressine at the highest concentrations significantly inhibited the adhesion to the same

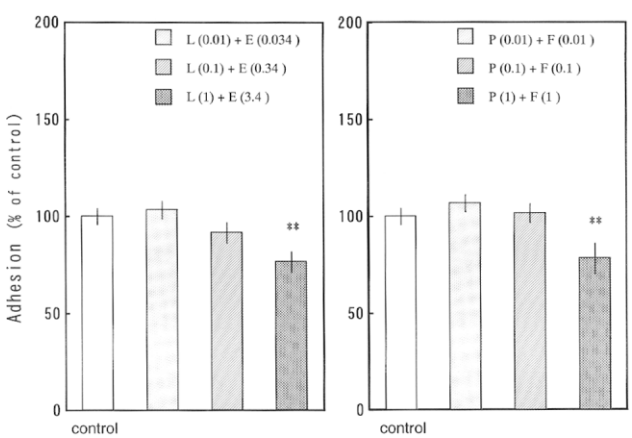

Fig. 1. Effects of two local anesthetics on adhesion in neutrophils. Neutrophils were incubated with lidocaine (L; mg/ml)–epinephrine (E; μM) and prilocaine (P; mg/ml)–felypressine (F; mU/ml) at one of three concentrations for 1 h. The data from eight separate experiment are shown. $^{**}P < 0.01$, significantly different from each control value obtained in neutrophils treated with medium.

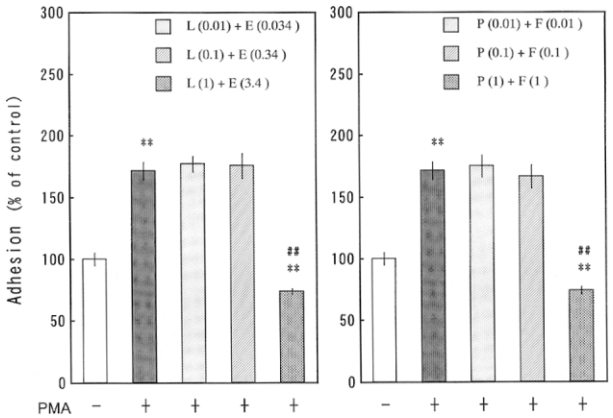

Fig. 2. Effects of two local anesthetics on adhesion in PMA-stimulated neutrophils. Neutrophils were incubated with lidocaine (L; mg/ml)–epinephrine (E; μM) and prilocaine (P; mg/ml)–felypressine (F; mU/ml) at one of three concentrations for 1 h in the presence of stimulated PMA at 200 nM. The data from four separate experiment are shown. $^{**}P < 0.01$, significantly different from each control value obtained in neutrophils treated with medium. $\#\,\#\,P < 0.01$, significantly different from each control value obtained in neutrophils treated with PMA alone.

level of the inhibition by these drugs in non-stimulated neutrophils in addition to completely preventing the potentiation by PMA. However, these drugs at other concentrations did not significantly affect the adhesion in PMA-stimulated neutrophils.

3.2. Mac-1

In order to determine whether the inhibition of adhesion indeed originates from the fluctuation of adhesion molecules, the expression of Mac-1 on neutrophils treated with both anesthetics was examined in flow cytometric assays. Mac-1 is a typical adhesion molecule on neutrophils and a heterodimeric complex consisting of CD11b and CD18. For determination, a FITC-conjugated monoclonal antibody against CD11b was used in the assays. Untreated neutrophils were found to express CD11b (Fig. 3a). Treatment with lidocaine at 0.1 mg/ml–epinephrine at 0.34 μM (data not shown) and lidocaine at 1 mg/ml–epinephrine at 3.4 μM (Fig. 3b) for 1 h inhibited the expression of CD11b on neutrophils in a dose-dependent manner. Prilocaine–felypressine was similarly effective in inhibiting the expression of CD11b (Fig. 3c). However, both anesthetics at the lowest concentration did not markedly affect expression of CD11b on neutrophils (data not shown). In contrast, PMA at 200 nM increased the expression of CD11b on neutrophils (Fig. 4a). However, both anesthetics at the highest concentrations failed to significantly affect the expression of CD11b on neutrophils (Fig. 4bc). Similarly, these drugs at the two lower concentrations did not markedly affect that (data not shown).

3.3. Phagocytosis

As shown in Table 1, lidocaine at 1 mg/ml–epinephrine at 3.4 μM caused almost 60% inhibition of phagocytosis of *E. coli* in neutrophils, in contrast, this combination at other doses failed to affect phagocytosis. Similarly, marked inhibition of phagocytosis occurred on the addition of prilocaine at 1 mg/ml–felypressine at 1 mU/ml. However, no significant alteration was detected in neutrophils on the addition of these at less than prilocaine at 1 mg/ml–felypressine at 1 mU/ml.

3.4. Superoxide anion production

Fig. 5 shows the effect of dental local anesthetics on the production of superoxide anion in the presence of stimulated PMA. The addition of lidocaine at 1 mg/ml–epinephrine at 3.4 μM caused almost a 40% increase in the production of superoxide anion 0.5 h after the incubation, in contrast, lidocaine at 0.1 mg/ml–epinephrine at 0.34 μM was effective in slightly but statistically potentiating the production (Fig. 5a). The potentiation was maximal 0.5 h after the incubation with a gradual recovery to the control level within 4 h. However, lidocaine–epinephrine at a lowest concentration was not effective as determined at different times after the incubation up to 4 h.

By contrast, prilocaine at 0.1 mg/ml–felypressine at 0.1 mU/ml markedly inhibited the production of superoxide anion 0.5 h after the incubation (Fig. 5b). Moreover, prilocaine at 1 mg/ml–felypressine at 1 mU/ml induced a more potent inhibition, which persisted for at least 4 h after the incubation. However, prilocaine–felypressine at a lowest concentration was not effective up to 4 h after the incubation.

3.5. Hydrogen peroxide production

Lidocaine–epinephrine significantly inhibited the production of hydrogen peroxide in proportion to increasing drug concentrations (Fig. 6, left panel). Namely, lidocaine–epinephrine at a highest dose caused almost a 70% decrease 15 min after the incubation with a gradual recovery within 60 min. In addition, the addition of prilocaine–felypressine similarly inhibited the production (Fig. 6, right panel).

3.6. Mechanisms

Although lidocaine-mediated inhibition of superoxide anion production by neutrophils stimulated with PMA for 0.5 h had been earlier demonstrated [15] that lidocaine–epinephrine could increase its production was a new and unexpected finding. Therefore, subsequent analysis focused on this potentiation of superox-

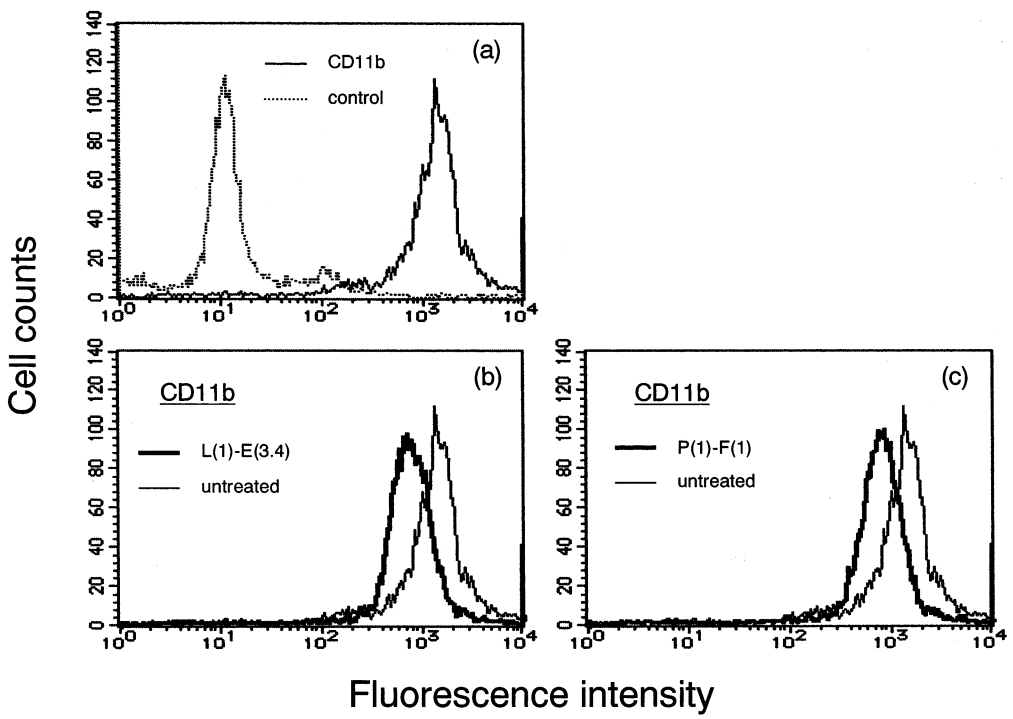

Fig. 3. Effects of two local anesthetics on Mac-1 expression in neutrophils. (a) Representative histogram of neutrophils staining with anti-CD11b antibody as compared with IgG control antibody. (b)(c) Representative histogram of neutrophils treated with lidocaine (L; mg/ml)–epinephrine (E; μM) and prilocaine (P; mg/ml)–felypressine (F; mU/ml) for 1 h as compared with untreated neutrophils staining with anti-CD11b antibody. Histograms from a typical experiment are shown.

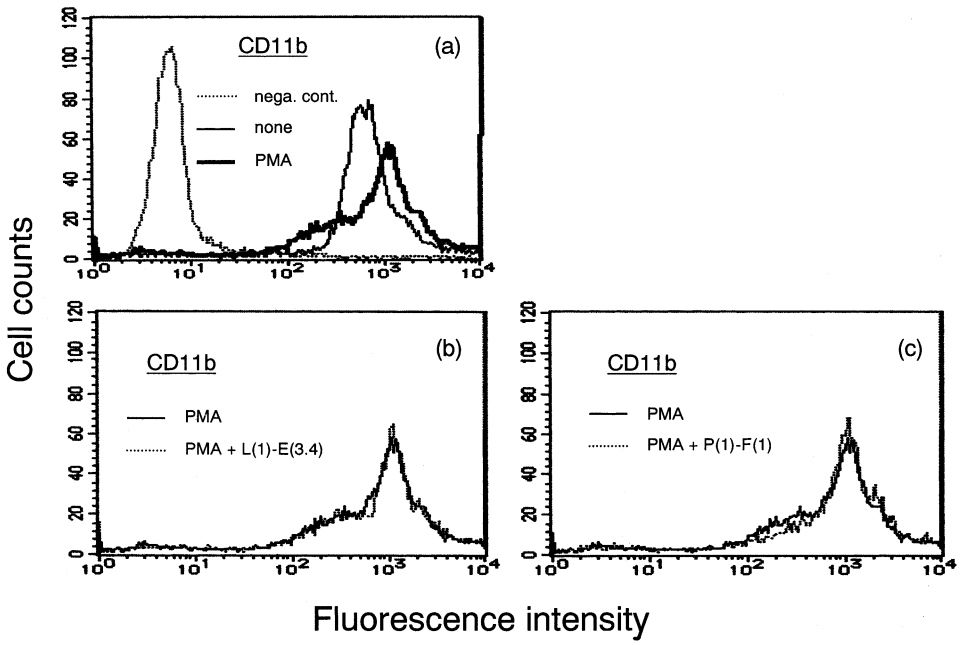

Fig. 4. Effects of two local anesthetics on Mac-1 expression in PMA-stimulated neutrophils. (a) Representative histogram of neutrophils in either the presence or absence of PMA at 200 nM staining with anti-CD11b antibody as compared with IgG control antibody. (b) and (c) Representative histogram of neutrophils treated with lidocaine (L; mg/ml)–epinephrine (E; μM) and prilocaine (P; mg/ml)–felypressine (F; mU/ml) for 1 h as compared with PMA-stimulated neutrophils staining with anti-CD11b antibody. Histograms from a typical experiment are shown.

ide anion production by lidocaine–epinephrine. In order to elucidate the possible involvement of adrenoceptor in the observed potentiation after incubation with lidocaine–epinephrine, the α-selective antagonist phentolamine and the β-selective antagonist propranolol were added to PMA-stimulated neutrophils together with lidocaine–epinephrine. However, neither phentolamine nor propranolol significantly affected the lidocaine–epinephrine induced potentiation of superoxide anion production after 0.5 h incubation (Fig. 7).

In order to further examine the specificity of the lidocaine–epinephrine induced potentiation of superoxide anion production by neutrophils, an attempt was made to determine whether the prilocaine together with epinephrine results in similar significant potentiation of superoxide anion production. According to the result, prilocaine together with epinephrine significantly inhibited the production of superoxide anion by neutrophils (Fig. 8). However, prilocaine–epinephrine was much less potent than prilocaine–felypressine in inhibiting the superoxide anion production.

4. Discussion

The essential importance of the findings presented in this study is that local anesthetics with blood vessel contraction drugs affect the neutrophil functions. This is the first direct demonstration of an interaction between local anesthetics with blood vessel contraction drugs and host defense functions of neutrophils. In this study, lidocaine–epinephrine evidently differs from prilocaine–felypressine in the molecular mechanisms underlying modulation of the production of superoxide anion in neutrophils. In fact, lidocaine–epinephrine induced transient potentiation of the production of superoxide anion, although both lidocaine and prilocaine inhibited the production of superoxide anion in neutrophils [15]. By contrast, prilocaine–felypressine induced persistent inhibition of that. Moreover, prilo-

Table 1
Effects of local anesthetics on phagocytosis of *Escherichia coli* by neutrophils[a]

		Fluorescent intensity (% of control)
Lidocaine (mg/ml)	*Epinephrine (μM)*	
0.01	0.034	102.7 ± 5.6
0.1	0.34	96.2 ± 7.4
1	3.4	39.3 ± 3.6**
Prilocaine (mg/ml)	*Felypressine (mU/ml)*	
0.01	0.01	101.5 ± 2.2
0.1	0.1	92.3 ± 4.2
1	1	39.8 ± 5.0**

[a] Neutrophil suspensions were adhered to plates for 1 h, followed by incubation with *Escherichia coli* containing the local anesthetics at one of three concentrations for 1 h. The data from six separate experiment are shown. $^{**}P<0.01$, significantly different from each control value obtained in neutrophils treated with medium.

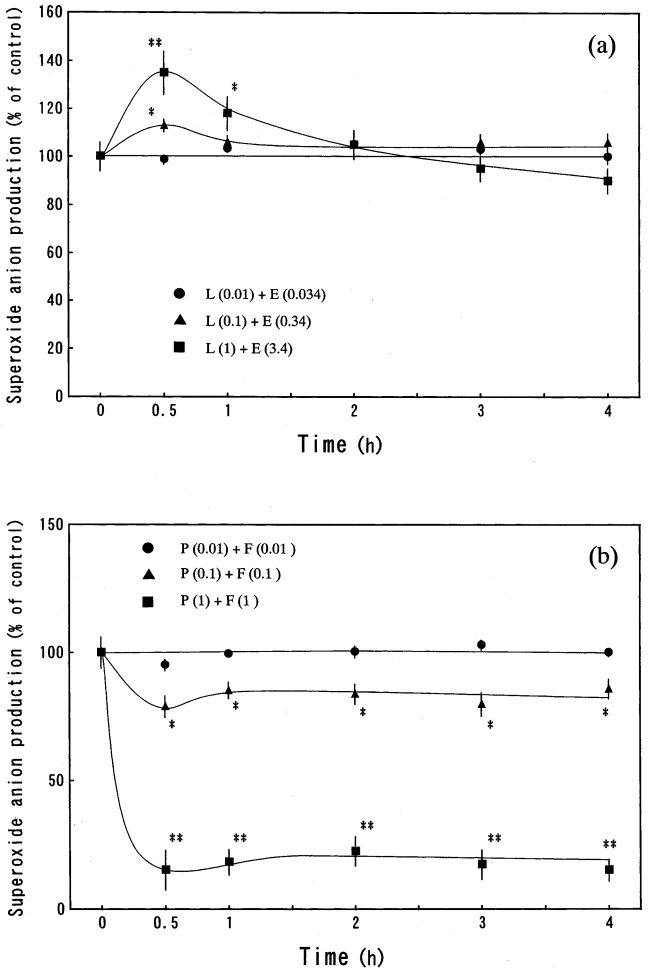

Fig. 5. Effects of (a) lidocaine (L; mg/ml)–epinephrine (E; μM) and (b) prilocaine (P; mg/ml)–felypressine (F; mU/ml) on superoxide anion production by neutrophils. Neutrophils were incubated with the local anesthetics at one of three concentrations in the presence of stimulated PMA. The data from 12 for (a) or four for (b) independent experiment are shown. $*P < 0.05$, $**P < 0.01$, significantly different from each control value obtained in neutrophils stimulated with PMA alone.

caine–felypressine inhibited the production of hydrogen peroxide concomitant with the inhibition of the production of superoxide anion, while lidocaine–epinephrine inhibited the production of hydrogen peroxide in spite of the potentiation of the production of superoxide anion. In addition, prilocaine–epinephrine was much less potent than prilocaine–felypressine in inhibiting the superoxide anion production. Therefore, it is likely that epinephrine appears to have some stimulatory activity of superoxide anion production which blunts the inhibitory activity of prilocaine. Furthermore, since α-selective antagonist phentolamine and β-selective antagonist propranolol did not inhibit the potentiation of the production of superoxide anion, epinephrine could stimulate the production of superoxide anion through a molecular mechanism different from a pathway via activation of adrenoceptors. However, it is unlikely that epinephrine produces the superoxide anion since lidocaine–epinephrine inhibited the hydrogen peroxide production in spite of the potentiation of superoxide anion production. For example, superoxide anion generated during respiratory burst is converted enzymatically into hydrogen peroxide by superoxide dismutase. The increase of superoxide anion production, in principle, would result in increase of hydrogen peroxide production. One possible explanation of this stimulatory activity is that epinephrine may

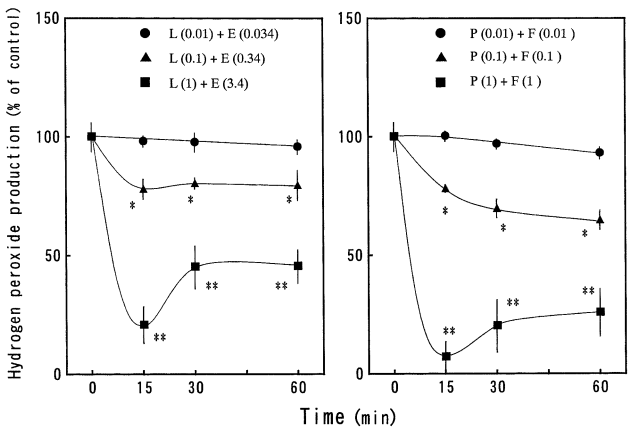

Fig. 6. Effects of lidocaine (L; mg/ml)–epinephrine (E; μM) and prilocaine (P; mg/ml)–felypressine (F; mU/ml) on hydrogen peroxide production by neutrophils. Neutrophils were incubated with the local anesthetics at one of three concentrations in the presence of stimulated PMA. The data are percentages of absorbance in the 600 nm wavelength in neutrophils treated with each agent over that of control neutrophils stimulated with PMA alone. Values are all from four to six independent experiment. $*P < 0.05$, $**P < 0.01$, significantly different from each control value obtained in neutrophils stimulated with PMA alone.

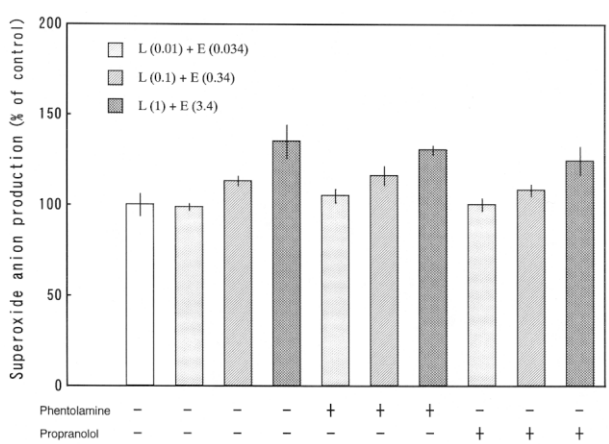

Fig. 7. Effects of selective adrenergic antagonists on lidocaine-epinephrine induced potentiation of superoxide anion production by neutrophils. Neutrophils were incubated with lidocaine (L; mg/ml)–epinephrine (E; μM) at one of three concentrations together with the α-selective antagonist phentolamine or the β-selective antagonist propranolol at a concentration of 10 μM for 0.5 h. The data from four to twelve separate experiment are shown.

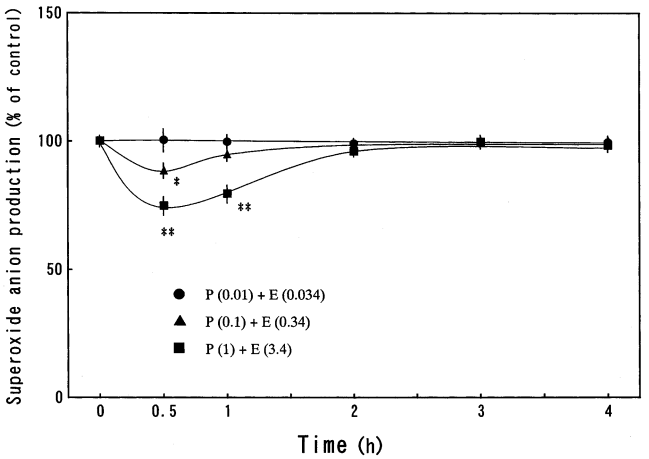

Fig. 8. Effects of prilocaine (P; mg/ml)–epinephrine (E; μM) on superoxide anion production by neutrophils. Neutrophils were incubated with prilocaine-epinephrine at one of three concentrations in the presence of stimulated PMA. The data from four independent experiments are shown. $*P < 0.05$, $**P < 0.01$, significantly different from each control value obtained in neutrophils stimulated with PMA alone.

inhibit superoxide dismutase, followed by inhibition of degradation of superoxide anion. In contrast, earlier findings have demonstrated that lidocaine possesses the ability to scavenge hydroxyl radical and singlet oxygen [16,17], but not hydrogen peroxide [18]. In addition, the possibility that local anesthetics are a scavenger of superoxide anion is available in the literature [19,20]. Furthermore, lidocaine decreased intracellular Ca^{2+} concentrations [21] and inhibited protein kinase C activity in murine brain [22]. However, local anesthetic inhibited the production of superoxide anion without affecting the phosphorylation of $p47^{phox}$ [23]. In neutrophils, whether local anesthetics inhibit protein kinase C activity is not clear. If lidocaine inhibits protein kinase C activity in neutrophils, lidocaine–epinephrine should inhibit the production of superoxide anion. These findings all give support to the idea that the molecular mechanisms underlying potentiation of the production of superoxide anion in neutrophils treated with lidocaine–epinephrine is associated with the inhibition of degradation of superoxide anion by inhibition of superoxide dismutase. In addition, it is conceivable that prilocaine–felypressine may inhibit the production of superoxide anion and hydrogen peroxide through scavenging the reactive oxygen species rather than the inhibition of protein kinase C.

The present study has demonstrated that both local anesthetics at their highest concentration inhibit adhesion and phagocytosis in neutrophils obtained 1 h after the incubation. In addition, both local anesthetics at their middle and highest concentrations induced an inhibition of adhesion molecule Mac-1 expression in neutrophils. In PMA-stimulated neutrophils, however, both local anesthetics at their highest concentration significantly inhibited adhesion, without affecting the Mac-1 expression. This means that both local anesthetics may inhibit adhesion in neutrophils through a molecular mechanism different from a pathway involving adhesion molecule Mac-1. In contrast, phagocytosis is the process by which cells recognize and engulf large particles and is important to host defense mechanisms as well as to tissue repair and morphogenetic remodeling. Furthermore, two of the best characterized phagocytic receptors in neutrophils, Fc gamma (Fcγ) receptors and the complement receptor 3 (CR3), and another named Mac-1, are involved in the uptake of microorganisms during infection. In fact, Mac-1 is associated with adhesion to endothelial cells and uptake of microorganisms. Fc receptors induce phagocytosis while complement receptors promote phagocytosis, but only if a second signal is provided by simultaneous binding via Fc receptors. Therefore, the inhibition of Mac-1 expression by both local anesthetics would be at least in part associated with the inhibition of phagocytosis, although both local anesthetics at the middle concentrations did not inhibit phagocytosis.

One interesting finding of this study is that there is a profound difference in modulation of the production of superoxide anion with local anesthetics treatment. Nonetheless, it is thus far unclear which local anesthetic is more effective in clinical use. However, it can be unequivocally concluded that lidocaine–epinephrine did induce transient potentiation of superoxide anion production, while prilocaine–felypressine persistently inhibited it. The longer the duration of production of superoxide anion, in principle, the more effective would be the sterilization of the invading bacteria at the site of inflammation. Therefore, it is possible to speculate that under clinical conditions, lidocaine–epinephrine may be more effective than prilocaine–felypressine for potentiating superoxide anion production (and therefore, microbicidal activity). In a earlier study, moreover, local anesthetics caused not only the modulation of the neutrophil functions in host defense, but also the inhibition of bacterial growth. For instance, local anesthetics are reported to possess antimicrobial activity, such as activity to inhibit of *Staphylococcus aureus* growth [24–29]. In particular, lidocaine at 10 mg/ml reduced the bacterial growth, while lidocaine at 2 mg/ml did not inhibit the bacterial growth [4]. It is likely that the using of local anesthetics at 1 mg/ml causes an increase of the infection rate or a delay of the recovery through the inhibition of neutrophil functions. Therefore, it is more conceivable that lidocaine–epinephrine at least in part may be effective for potentiating superoxide anion production.

It thus appears that lidocaine–epinephrine and prilocaine–felypressine differentially modulate the production of superoxide anion, and could similarly affect

adhesion, phagocytosis, and the production of hydrogen peroxide by neutrophils. In addition, lidocaine and prilocaine both inhibited adhesion, phagocytosis, and the production of superoxide anion and hydrogen peroxide by neutrophils in a similar fashion as shown earlier [15]. Elucidation of the interaction among local anesthetics, host defense and bacterium needs to be evaluated in future studies, and undoubtedly will have great benefits for therapy in humans.

Acknowledgements

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