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LIDOCAINE INHIBITS PRIMING AND PROTEIN TYROSINE PHOSPHORYLATION OF HUMAN PERIPHERAL NEUTROPHILS

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Abstract—The addition of agents, such as tumor necrosis factor-α, to human peripheral neutrophils (HPPMN) induces priming, which enhances the receptor-mediated superoxide (O_2^-) generation and tyrosine phosphorylation of several HPPMN proteins. Lidocaine, a local anesthetic, inhibited both enhanced O_2^- generation and tyrosine phosphorylation of a 115 kDa protein in a concentration- and time-dependent manner. Lidocaine also inhibited protein kinase C sensitive O_2^- generation induced by phorbol myristate acetate, but not time dependently. Furthermore, lidocaine inhibited O_2^- generation by non-primed HPPMN induced by formylmethionyl-leucyl-phenylalanine, but this inhibition needed a higher concentration of lidocaine compared with that of primed HPPMN. These results suggest that lidocaine inhibits the priming step of neutrophil activation and that it is linked to the inhibition of tyrosine phosphorylation of a 115 kDa protein.

Neutrophils play an important role in host defense mechanisms and inflammatory responses [1], but in the peripheral blood of healthy humans, they are relatively inactive. Several agents, such as tumor necrosis factor-α (TNF-α†) and granulocyte colony-stimulating factor [2, 3], activate neutrophils and enhance several responses including an increased capacity to produce superoxide anions (O_2^-) [4, 5], adherence to endothelial cells [6], migration [5] and lysosomal enzyme release [4, 7]. These enhanced neutrophil responses are termed "priming". Primed neutrophils *in vivo* play a major role in protecting the host from invading microorganisms by generating several active oxygen species that kill microorganisms [1]. When neutrophils respond to foreign bodies, NADPH oxidase is activated through signal transduction pathways and catalyses the one-electron reduction of oxygen to O_2^- followed by the generation of several other active oxygen species [8].

Lidocaine is a widely clinically used local anesthetic. In addition to its anesthetic action, it has several effects on neutrophil functions, such as inhibition of chemotaxis [9, 10] and phagocytosis [11, 12], and reduction of lysosomal enzyme release and O_2^- production [13, 14]. There is a good correlation between anesthetic potency and lipid solubility [15] and local anesthetics affect biological membranes in a variety of ways [16]. We questioned whether local anesthetics show these inhibitory actions upon cellular membrane phospholipid, and studied the effects of lidocaine on stimulation-coupled responses and protein kinase C (PKC) activity in guinea pig peritoneal neutrophils, primed *in vivo*. We found that the decrease in O_2^- generation caused by lidocaine correlates with an inhibition of PKC activity [17].

Recent reports revealed that priming caused by various agents is accompanied by tyrosine phosphorylation of neutrophil proteins [18–21], and this phosphorylation may be involved in signaling pathways other than those mediated by PKC [22, 23]. Thus, we conducted a study to clarify the effect of lidocaine on the priming of human peripheral neutrophils (HPPMN) and tyrosine phosphorylation of the proteins of HPPMN.

MATERIALS AND METHODS

Chemicals. Ferricytochrome *c* (Cyt.c), formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Lidocaine hydrochloride was donated from the Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan). Human TNF-α was donated by Hayashibara Bioscience Research Institute (Okayama, Japan). Anti-phosphotyrosine antibody (PY-20) and peroxidase-labeled anti-mouse immunoglobulin antibody were purchased from ICN (Irvin, CA, U.S.A.) and CAPPEL (Durham, NC, U.S.A.), respectively. All other agents were of analytical grade and were obtained from the Nacalai Tesque Co. (Kyoto).

Preparation and priming of neutrophils. HPPMN were isolated from the fresh blood of healthy volunteers by the method of Ficoll/Hypaque gradients [24]. Their isolated neutrophils were washed twice with Krebs–Ringer phosphate (KRP) and resuspended at a concentration of 1×10^8 cells/

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† Abbreviations: Cyt. c, ferricytochrome *c*; FMLP, formylmethionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; PKC, protein kinase C; HPPMN, human peripheral neutrophils; KRP, Krebs–Ringer phosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-α.

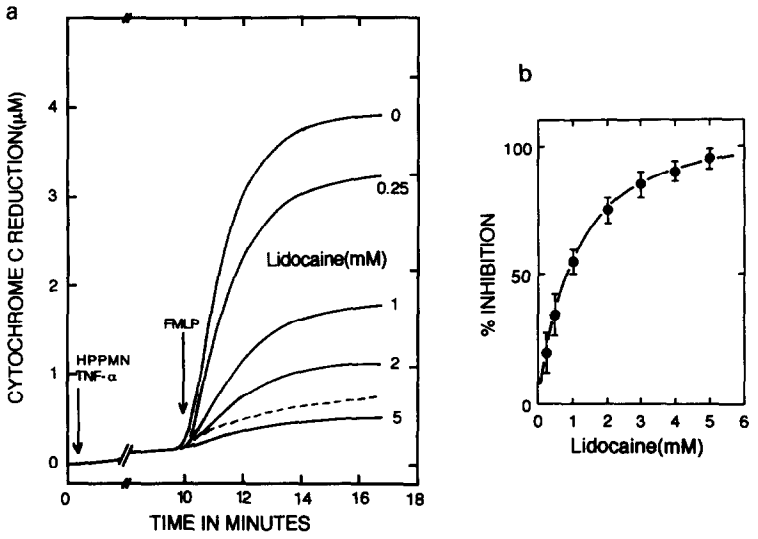

Fig. 1. Effect of lidocaine on FMLP-induced O_2^- generation by TNF-α-treated HPPMN. HPPMN (1×10^6 cells/mL) were incubated in 2 mL KRP containing 1 mM $CaCl_2$, 10 mM glucose, 20 mM Cyt.c, in the presence or absence of 2 U/mL TNF-α at 37°. Lidocaine was added 10 sec after TNF-α. O_2^- generation was triggered by adding 1.25×10^{-8} M FMLP after a 10-min incubation with TNF-α. The change in absorbance at 550–540 nm ($A_{550-540}$) was monitored and O_2^- generation was calculated from superoxide dismutase-inhibitable Cyt.c reduction. (a) Traces of O_2^- generation in the presence of various concentrations of lidocaine. The broken line shows FMLP-induced O_2^- generation in the absence of TNF-α. (b) Dose–response curves for lidocaine inhibition. Results are means ± SE from three different experiments. Numbers in the figure show the concentration of lidocaine (mM).

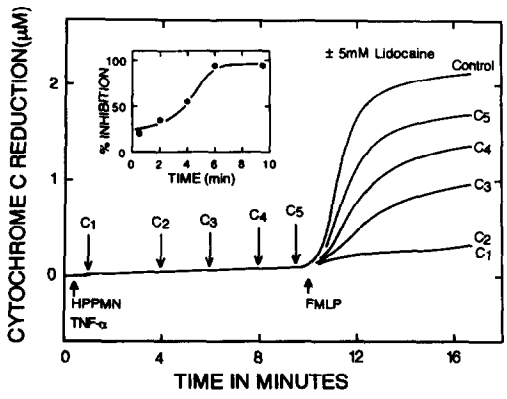

Fig. 2. Effect of preincubation with lidocaine on FMLP-induced O_2^- generation by TNF-α-treated HPPMN. Experimental conditions were the same as those described in the legend to Fig. 1. Lidocaine (5 mM) was added at different times after the addition of TNF-α. Control shows O_2^- generation without the addition of lidocaine. C_1–C_5 show the time of lidocaine addition before treatment with FMLP. Inset shows the time-dependent inhibition curve for lidocaine.

mL. Cell viability was determined by Trypan blue exclusion. HPPMN (1×10^6 cells/mL) were primed with 2 U/mL of TNF-α.

Measurement of O_2^- generation. O_2^- generation was assayed by Cyt.c reduction, using a dual beam spectrophotometer (Shimadzu UV 3000). Briefly, the reaction mixture contained in a final volume of 2 mL KRP, 1 mM $CaCl_2$, 20 mM Cyt.c, 10 mM glucose, 1.5 mM NaN_3 and 1×10^6 cells/mL. TNF-α 2 U/mL, was added as the priming compound. In the presence and absence of various concentrations of lidocaine, reactions were started by adding a ligand and the change in absorbance at 550–540 nm ($A_{550-540}$) was monitored at 37°. Generation of O_2^- was calculated from the rate of O_2^- dismutase-inhibitable Cyt.c reduction using an extinction coefficient of 21 mM^{-1} [25].

Detection of phosphorylated tyrosine in neutrophil proteins. Phosphorylated tyrosine was detected by immunoblotting with a monoclonal antibody for phosphotyrosine. HPPMN (2×10^6 cells/mL) suspended in KRP were incubated with 10 U/mL TNF-α at 37° in the presence or absence of lidocaine. The reaction was stopped by adding ice-cold 15% trichloroacetic acid containing 2 mM phenyl methylsulfonyl fluoride and 1 mM sodium orthovanadate. The precipitate was washed twice with ice-cold ether/ethanol (1:1), dissolved in SDS sample buffer, and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, proteins were transferred to a Immobilon-P filter (Millipore) in a Sartorius semi-dry blotter. The filter was incubated for 60 min in 30 mM Tris–HCl (pH 7.5)-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% skim milk at room temperature. The filter was incubated with phosphotyrosine-specific monoclonal antibody (PY-20, 1/3000 diluted in TBS-T containing 0.5% skim milk).

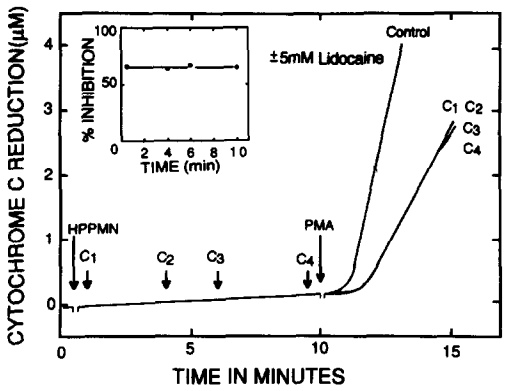

Fig. 3. Effect of preincubation with lidocaine on PMA-induced O_2^- generation by HPPMN. Experimental conditions were the same as those described in the legend to Fig. 2. O_2^- generation was induced by adding 1×10^{-9} M PMA; the O_2^- generation was inhibited by 65% with 5 mM lidocaine. This inhibitory effect of lidocaine was not changed by the duration of exposure.

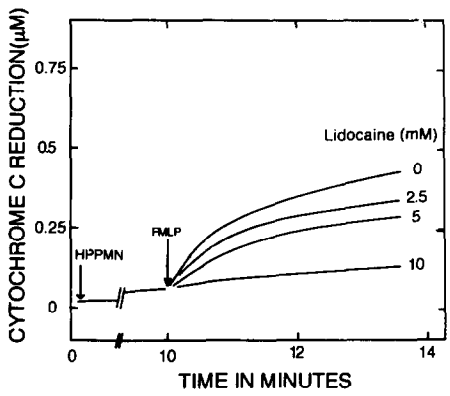

Fig. 4. Effect of lidocaine on FMLP-stimulated superoxide generation by HPPMN without priming by TNF-α. Experimental conditions were the same as those described in the legend to Fig. 1. The density of HPPMN was increased to 3×10^6 cells/mL and FMLP was also increased to 2.5×10^{-8} M; O_2^- generation by non-primed HPPMN was very low regardless of the increased number of incubated cells and FMLP. Lidocaine inhibited O_2^- generation at a higher concentration than that described in Fig. 1.

The monoclonal antiphosphotyrosine antibody was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin. Peroxidase positive bands were detected using the ECL western blotting detection system (Amersham). Molecular masses were determined using Daiichi-Kagaku standards.

RESULTS

Enhancement of receptor-mediated active oxygen generation in HPPMN by TNF-α and its inhibition by lidocaine

It has been reported that FMLP-stimulated O_2^- generation is markedly enhanced by priming with TNF-α, in a concentration- and time-dependent manner [2, 3]. We confirmed these findings and further demonstrated that the half maximal concentration of TNF-α was 2 U/mL, and that the required time to obtain maximal stimulation was about 10 min [20, 21]. Lidocaine inhibited the enhanced O_2^- generation induced by TNF-α in a concentration-dependent manner (Fig. 1) and, at 5 mM, caused 95% inhibition of O_2^- generation. The concentration of lidocaine for half maximum inhibition of O_2^- generation was 0.75 mM.

Incubation time-dependent inhibition of TNF-α-primed FMLP-stimulated O_2^- generation by lidocaine

To gain further insight into the mechanism of inhibition by lidocaine, the effect of incubation time with lidocaine on TNF-α-induced priming was investigated. As shown in Fig. 2, the inhibition rate of lidocaine was reduced from 95 to 20% by decreasing the incubation time with lidocaine. This time dependence is shown in the inset of Fig. 2. When lidocaine was added at 30 sec or 4 min after incubation with TNF-α, no enhancement of O_2^- generation with FMLP was observed. However, the inhibitory effect was greatly reduced by adding lidocaine 9.5 min after exposure to TNF-α.

Effect of lidocaine on PMA-stimulated O_2^- generation by HPPMN

Lidocaine reduced PMA-stimulated O_2^- generation to 65% at 5 mM. However, the rate of inhibition of lidocaine was not changed by the time of incubation (Fig. 3).

Effect of lidocaine on FMLP-stimulated O_2^- generation by non-primed HPPMN

Lidocaine inhibited O_2^- generation of non-primed HPPMN at a high concentration. The concentration for half maximum inhibition of O_2^- generation was about 5 mM (Fig. 4), which was much higher than that required for TNF-α-primed FMLP-induced O_2^- generation.

Enhancement of tyrosine phosphorylation of neutrophil proteins by TNF-α priming and inhibition of this phosphorylation by lidocaine

The tyrosine phosphorylation of 84, 108 and 115 kDa proteins occurred in the presence of TNF-α (Fig. 5 lane 2). Lidocaine inhibited tyrosine phosphorylation of these neutrophil proteins in a concentration-dependent manner (Fig. 5). When HPPMN were incubated with 3 mM lidocaine for 9.5 min following the addition of TNF-α, tyrosine phosphorylation of the 115 kDa protein did not occur. However, this inhibition decreased with reduced incubation period (Fig. 6). The inhibition depended on the length of the incubation with lidocaine.

DISCUSSION

TNF-α is a cytokine with various biological effects [26]. Priming of HPPMN with TNF-α remarkably enhanced FMLP-stimulated O_2^- generation in a concentration- and time-dependent manner. The

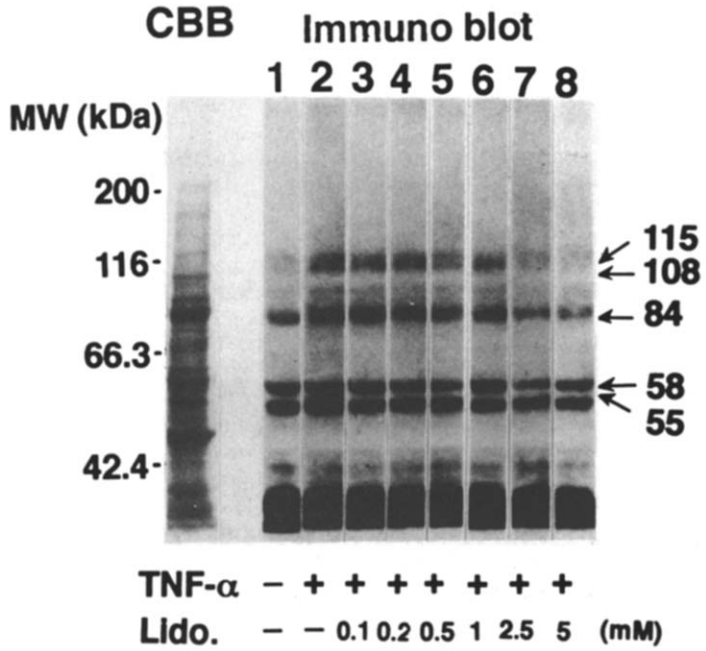

Fig. 5. Effect of TNF-α on neutrophil protein tyrosine phosphorylation and its inhibition by lidocaine. HPPMN (2×10^6 cells/mL) were incubated at 37° in KRP medium containing 1 mM $CaCl_2$, 10 mM glucose and 10 U/mL TNF-α in the absence or presence of various concentrations of lidocaine. The reaction was stopped with 15% trichloroacetic acid and SDS–PAGE was performed in the presence of phenylmethyl sulfonyl fluoride and vanadate. After transfer to an Immobilon-P filter, proteins were immunoblotted with monoclonal antiphosphotyrosine antibody and the antibody was detected with the ECL western blotting detection system. Phosphorylation of 84, 108 and 115 kDa proteins was inhibited by lidocaine in a concentration-dependent manner. CBB, stained with Coomassie brilliant blue; Immunoblot, immunoblotting with antiphosphotyrosine antibody; Lido, lidocaine concentration (mM).

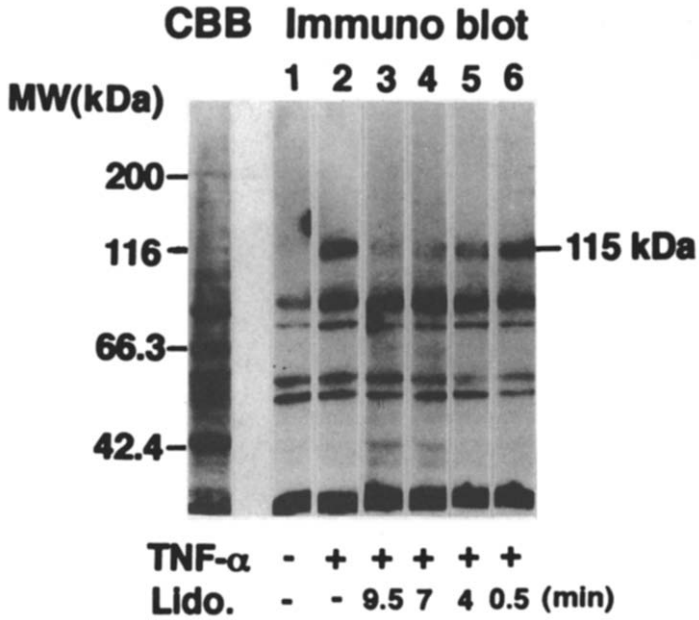

Fig. 6. Effect of preincubation with lidocaine on tyrosine phosphorylation in TNF-α-treated neutrophils. Experimental conditions were the same as those described in the legend to Fig. 5. The duration of exposure to lidocaine was changed from 30 sec to 9.5 min.

concentration of TNF-α required to obtain maximum stimulation was about 10 U/mL and the incubation time was about 10 min (data not shown). However, TNF-α enhancement slightly varied among individuals. We investigated its effect at a concentration of 2 U/mL (Fig. 1). TNF-α increased tyrosine phosphorylation in several cells [27, 28]. The mechanism of signal transduction by TNF-α involves activation of tyrosine kinase [27]. Stimulus-coupled O_2^- generation depends on the phosphorylation of specific proteins, and PKC activation plays an important role in the mechanism of stimulus-coupled O_2^- generation [29]. However, stimulus-coupled O_2^- generation is only partially inhibited by PKC inhibitors [30, 31]. These findings suggest that other protein kinases are involved in stimulus-coupled O_2^- generation. In addition, recent studies have suggested that tyrosine phosphorylation of several neutrophil proteins is involved in the priming process [18–21]. That is, several priming agents induced a time- and concentration-dependent increase in the tyrosine phosphorylation of several similar neutrophil proteins, including the 115 kDa protein [18–21]. This protein tyrosine phosphorylation is correlated with FMLP-dependent O_2^- generation [32]. Furthermore, tyrosine kinase inhibitors, such as genistein and ST 638 (α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide), inhibit both phosphorylation and O_2^- generation [3, 20, 21]. These findings indicate that tyrosine phosphorylation of neutrophil proteins, such as the 115 kDa protein, is involved in neutrophil priming.

Lidocaine inhibits the O_2^- generation by FMLP and PMA of primed guinea pig peritoneal neutrophils [17]. HPPMN in this study behaved similarly. Lidocaine may inhibit both receptor- and PKC-mediated O_2^- generation. When HPPMN were treated with lidocaine during priming with TNF-α, FMLP-dependent O_2^- generation was highly inhibited (Fig. 1), the rate of which depended on the length of exposure to lidocaine (Fig. 2). A higher concentration of lidocaine was required to inhibit O_2^- generation by FMLP of non-primed HPPMN (Fig. 4). There was no time-dependent inhibition of PMA-induced O_2^- generation (Fig. 3). These results suggest that lidocaine inhibits at the priming step of neutrophil activation. Furthermore, lidocaine inhibited the TNF-α-induced tyrosine phosphorylation of a 115 kDa protein specific to HPPMN. The inhibition of O_2^- generation by lidocaine was similar to that of the tyrosine phosphorylation of the 115 kDa protein and was concentration and time dependent. Thus, lidocaine may inhibit TNF-α-induced priming of neutrophils through inhibition of tyrosine phosphorylation.

In conclusion, lidocaine inhibits the TNF-α-induced priming process and enhances O_2^- generation, together with the tyrosine phosphorylation of the 115 kDa neutrophil protein. This suggests involvement of tyrosine phosphorylation in the neutrophil-priming process.

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