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Selective Inhibition of Formyl-Methionyl-Leucyl-Phenylalanine (fMLF)-Dependent Superoxide Generation in Neutrophils by Pravastatin, an Inhibitor of 3-Hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) Reductase

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ABSTRACT. It has been shown previously that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, such as compactin, lovastatin, and pravastatin, block cholesterol synthesis, suppress lymphocyte functions, and beneficially affect atherogenesis. Recently, it was reported that compactin and lovastatin inhibit the respiratory burst of DMSO-differentiated HL-60 cells, an effect reversed by mevalonic acid. The mode of action of these inhibitors in this role is not understood fully. Thus, we studied the mechanism of inhibition of neutrophil superoxide ($O_2^{\cdot-}$) generation by pravastatin and found that pravastatin at 0.5 mM inhibited the receptor-mediated tyrosine kinase (TK)-dependent pathway of $O_2^{\cdot-}$ generation and also luminol chemiluminescence but not the protein kinase C (PKC)-dependent or the TK- and PKC-independent pathways of $O_2^{\cdot-}$ generation in neutrophils. Pravastatin also inhibited the tumor necrosis factor-α- and formyl-methionyl-leucyl-phenylalanine-induced phosphorylation of a tyrosine of a 115-kDa protein. These effects were not reversed by mevalonate. From these results it is concluded that pravastatin inhibited receptor-mediated $O_2^{\cdot-}$ generation by decreasing tyrosine phosphorylation but not by inhibiting the formation of an intermediate in the biosynthesis of cholesterol. BIOCHEM PHARMACOL **58**;12:1975–1980, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. HMG-CoA reductase inhibitor; pravastatin; neutrophils; $O_2^{\cdot-}$ generation; tyrosine phosphorylation

HMG-CoA†† reductase is the enzyme at the rate-limiting step in cholesterol biosynthesis. Thus, inhibitors of HMG-CoA reductase have been used for therapeutic benefit in hypercholesterolemia patients [1]. Furthermore, it has been reported that intermediates of cholesterol synthesis, such as farnesylpyrophosphate and geranylgeranylpyrophosphate, are required for the isoprenylation of many membrane-bound proteins. Protein isoprenylation is a post-translational modification essential for the membrane localization and biological activity of a number of proteins [2–4]. The precursor of isoprenoid lipids involved in protein modification, as well as in the synthesis of cellular sterols, is mevalonic acid [5]. Isoprenylated proteins, among which are all the GTP-binding proteins, play crucial roles in the signal transduction pathways from growth factors, cytokines, and hormone receptors [6–9].

Treatment of DMSO-differentiated HL-60 cells with compactin, an inhibitor of HMG-CoA reductase, produces a concentration-dependent inhibition of $O_2^{\cdot-}$ formation elicited by incubation of cells with fMLF or PMA [10]. The inhibitory effect of compactin is specifically prevented by the addition of exogenous mevalonic acid to the culture medium of HL-60 cells, indicating that the inhibitory effects of the drug are due to blockade of the pathway leading to isoprenoid synthesis [10]. These data suggest that an isoprenoid pathway intermediate is required for activation of the NADPH oxidase of the phagocyte. Furthermore, a GTP-binding protein known as Rap1A has been shown to bind to the cytochrome *b* component of NADPH oxidase [11, 12]. In addition, a 22-kDa protein present in neutrophil cytosol and exhibiting oxidase-stimulating activity has

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†† *Abbreviations*: fMLF, formyl-methionyl-leucyl-phenylalanine; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; KRP, Krebs–Ringer–phosphate buffer; LCL, luminol chemiluminescence; $O_2^{\cdot-}$, superoxide; OZ, opsonized zymosan; PKC, protein kinase C; PI3-K, phosphatidylinositol-3-kinase; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; TK, tyrosine kinase; and TNF-α, tumor necrosis factor-α.

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been identified as another GTP-binding protein, Rac2 [13–15].

McGuire *et al.* [16] reported that lovastatin, an inhibitor of HMG-CoA reductase, disrupts early events in the mitogenic signaling pathway of insulin through the inhibition of PI3-K activity by preventing tyrosine phosphorylation of the β subunit of the insulin receptor. In previous papers, Utsumi and co-workers showed that genistein, an inhibitor of TK, suppresses fMLF-dependent $O_2^{\cdot-}$ generation in neutrophils by preventing tyrosine phosphorylation of a 115-kDa protein [17, 18]. The 115-kDa protein is the product of the human c-*cbl* proto-oncogene and activates PI3-K by tyrosine phosphorylation of the regulatory subunit [19]. Here we show that pravastatin, an inhibitor of HMG-CoA reductase, suppressed $O_2^{\cdot-}$ generation in neutrophils stimulated by fMLF by preventing tyrosine phosphorylation of a 115-kDa protein and that the inhibition was not reversed by mevalonic acid.

MATERIALS AND METHODS

Chemicals

Ferricytochrome *c*, fMLF, PMA, zymosan, and sodium arachidonate were purchased from the Sigma Chemical Co. Anti-phosphotyrosine monoclonal antibodies (PY-99) were obtained from Santa Cruz Biotechnology, Inc. Pravastatin was donated by the Sankyo Pharmaceutical Co. Ltd. fMLF and PMA were dissolved in ethanol, and the final concentration of ethanol in the reaction mixtures was less than 0.5%.

Cells

Human PMN were isolated from the peripheral blood of healthy donors using Polymorphoprep (Nycomed) [20]. PMN were stimulated by either 25 nM fMLF, 0.1 nM PMA, 200 μg/mL of OZ, or 30 μM sodium arachidonate at 37°. Preincubation of PMN with TNF-α for 10 min enhances fMLF-induced $O_2^{\cdot-}$ generation [21].

Measurement of $O_2^{\cdot-}$ Generation

Cellular generation of $O_2^{\cdot-}$ was assayed by measuring the reduction of ferricytochrome *c* using a dual beam spectrophotometer (Shimadzu UV-3000) equipped with a water-jacketed cell holder and magnetic stirrer [21]. Briefly, the reaction was started by adding 1×10^6 PMN/mL at 37° in 2 mL of KRP containing 10 mM glucose, 20 μM ferricytochrome *c*, and 1 mM $CaCl_2$ in the presence or absence of various reagents. Changes in absorbance at 550–540 nm ($A_{550-540}$) were monitored continuously.

Measurement of LCL

Chemiluminescence experiments were performed using a Luminescence Reader (Aloka BRL-201) or a calcium analyzer (Jasco CAF 100) [22]. The reaction mixture contained, in a final volume of 1 mL of KRP, 100 μM luminol, 1×10^6 cells/mL, and other additions. The intensity of LCL was recorded for 10–15 min.

Western Blot Analysis

Cell lysates were prepared as described elsewhere [17]. Cells (1×10^6/mL) were dissolved in 20 μL of SDS–sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.002% bromophenol blue) and boiled for 5 min. Then the samples were subjected to SDS–PAGE. After transfer of proteins in the gel to PVDF membrane (Immobilon-P^{TM}, Millipore Co.), the filter was incubated with primary antibody (1:2000 dilution) and then with horseradish peroxidase linked to secondary antibody (1:2000 dilution) and was analyzed by using an enhanced chemiluminescence kit (Amersham Co.).

Number of Experiments

All experiments were carried out more than three times, and representative figures are presented from similar results.

RESULTS

Effect of Pravastatin on $O_2^{\cdot-}$ Generation in Neutrophils

The effect of pravastatin on PMA- and fMLF-dependent $O_2^{\cdot-}$ generation in PMN was examined. PMN in peripheral blood are not primed. Thus, treatment with TNF-α was required for induction of $O_2^{\cdot-}$ generation by fMLF [20]. Pravastatin inhibited fMLF-induced but not PMA-induced $O_2^{\cdot-}$ generation (Fig. 1).

Selective Inhibition of TK-Sensitive $O_2^{\cdot-}$ Generation in PMN by Pravastatin

Figure 2 shows $O_2^{\cdot-}$ generation in PMN elicited by different stimuli and its suppression by different inhibitors. fMLF-induced $O_2^{\cdot-}$ generation was suppressed by genistein, an inhibitor of TK (data not shown). Similar to genistein, pravastatin inhibited fMLF-induced $O_2^{\cdot-}$ generation. The inhibited $O_2^{\cdot-}$ generation then was stimulated by PMA, and the resulting $O_2^{\cdot-}$ generation was suppressed by staurosporine, an inhibitor of PKC. $O_2^{\cdot-}$ generation was induced again by arachidonic acid and inhibited by cetylamine, a cationic amphiphile (Fig. 2). These results suggested that pravastatin inhibited only fMLF-dependent $O_2^{\cdot-}$ generation but not PKC-dependent or TK- and PKC-independent $O_2^{\cdot-}$ generation.

Effect of Pravastatin on OZ-Stimulated $O_2^{\cdot-}$ Generation

To test the selective inhibition of $O_2^{\cdot-}$ generation, the effect of pravastatin on OZ-induced $O_2^{\cdot-}$ generation was examined. Utsumi and co-workers reported previously that OZ-induced $O_2^{\cdot-}$ generation is a TK-sensitive reaction and

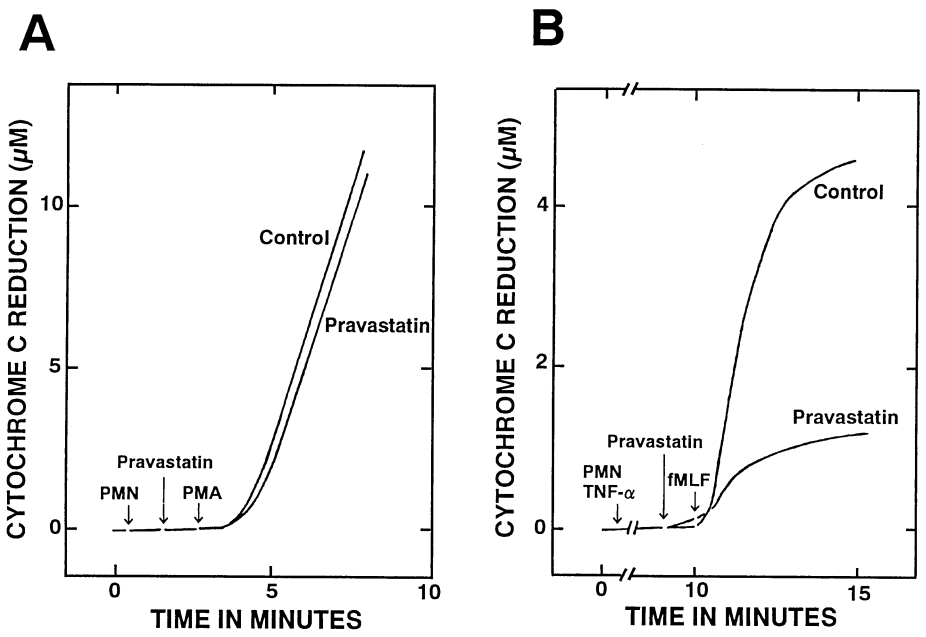

FIG. 1. Effect of pravastatin on $O_2^{\cdot -}$ generation in PMN. The reaction mixture contained, in a final volume of 2 mL of KRP (pH 7.4), 1 mM $CaCl_2$, 10 mM glucose, and 1 × 10^6 cells/mL. Ferricytochrome c reduction was measured spectrophotometrically at 550–540 nm and 37°. Final concentrations of PMA, TNF-α, fMLF, and pravastatin were 0.1 nM, 20 U/mL, 25 nM, and 0.5 mM, respectively. (A) PMA-stimulated respiratory burst; (B) fMLF-stimulated respiratory burst.

is inhibited by genistein [17, 18]. Consistent with the inhibition of fMLF-induced $O_2^{\cdot -}$ generation, OZ-induced $O_2^{\cdot -}$ generation was inhibited by pravastatin. The results indicated that pravastatin inhibited the TK-sensitive step of $O_2^{\cdot -}$ generation.

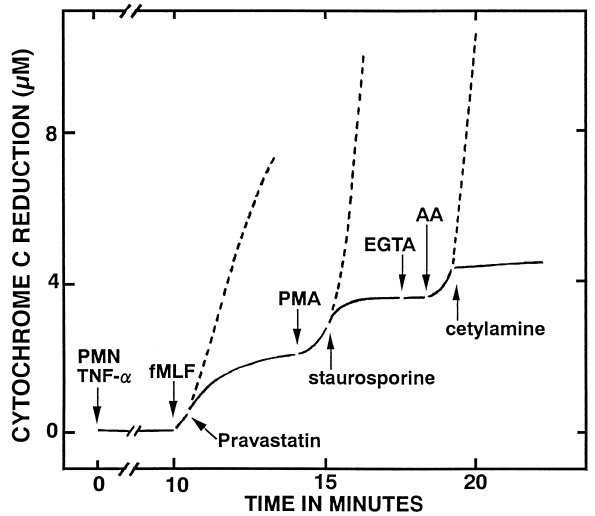

FIG. 2. Effect of pravastatin on $O_2^{\cdot -}$ generation elicited by specific stimuli and on its suppression by specific inhibitors. Experimental conditions were as in Fig. 1. The broken line shows the control experiment in the absence of inhibitors. Final concentrations of TNF-α, fMLF, PMA, arachidonate, and EGTA were 20 U/mL, 25 nM, 0.1 nM, 30 μM, and 1 mM, respectively. The concentrations of the inhibitors pravastatin, staurosporine, and cetylamine were 1 mM, 100 nM, and 100 μM, respectively.

Kinetic Analysis of Inhibitory Effect of Pravastatin on fMLF-Induced $O_2^{\cdot -}$ Generation

To obtain the concentration required for half-maximal inhibition, the effect of various concentrations of pravastatin on fMLF-induced $O_2^{\cdot -}$ generation was examined. The IC_{50} was about 0.4 mM (Fig. 4A). This effect was not reversed by mevalonic acid (Fig. 4B). The effects of the addition of pravastatin before or after the stimulation of

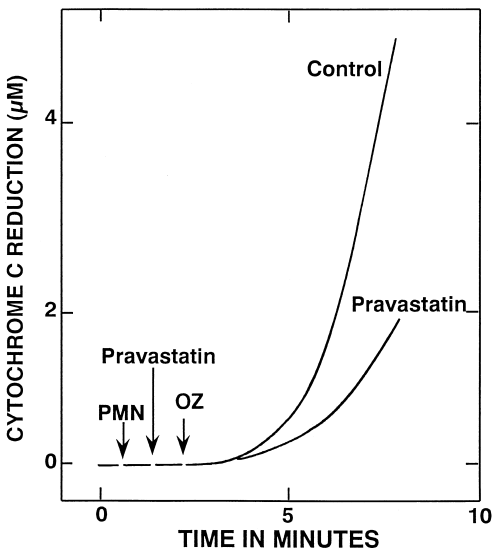

FIG. 3. Effect of pravastatin on OZ-induced $O_2^{\cdot -}$ generation in PMN. Experimental conditions were as in Fig. 1. Final concentrations of pravastatin and OZ were 0.5 mM and 200 μg/mL, respectively.

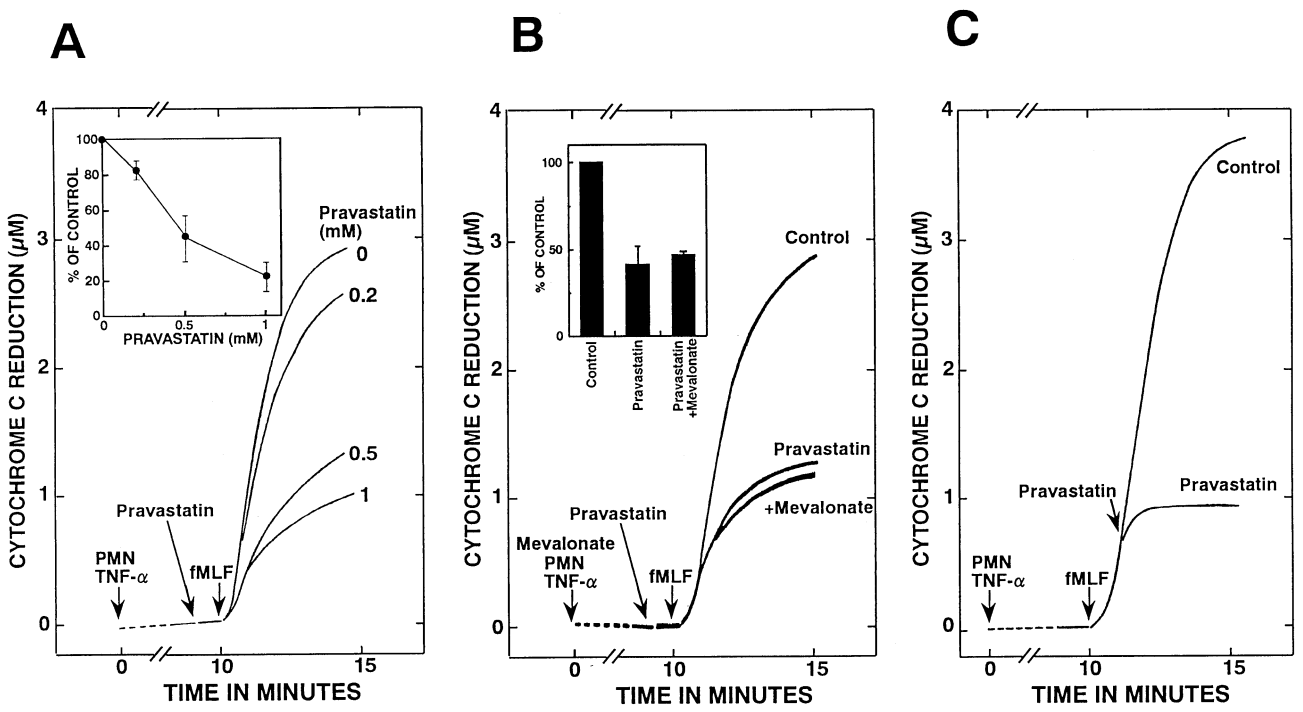

FIG. 4. Effect of pravastatin and mevalonic acid on fMLF-induced $O_2^{\cdot -}$ generation in PMN. Experimental conditions were as in Fig. 1. Final concentrations of TNF-α and fMLF were 20 U/mL and 25 nM, respectively. (A) Kinetic analysis of the inhibitory effect of pravastatin on fMLF-induced $O_2^{\cdot -}$ generation. (B) Effect of preincubation with mevalonic acid on the inhibition of neutrophil $O_2^{\cdot -}$ generation by pravastatin. (C) Effect of pravastatin (0.5 mM) on $O_2^{\cdot -}$ generation after treatment with fMLF. Data in the insets are the means ± SD of at least three independent experiments.

$O_2^{\cdot -}$ generation in PMN by fMLF were investigated. Pravastatin inhibited the $O_2^{\cdot -}$ generation when added immediately after treatment with fMLF, as it did when added prior to fMLF (Fig. 4C). These results indicate that pravastatin may inhibit TK activity.

Inhibition of fMLF-Induced LCL

PMN generated LCL by treatment with fMLF, and the LCL was decreased strongly by treatment with pravastatin (Fig. 5). It is well known that LCL responds to various reactive oxygen species, such as $O_2^{\cdot -}$, H_2O_2, $\cdot OH$, and OCl^-. In a previous paper, Utsumi and co-workers showed that fMLF-induced LCL is decreased by a small amount of superoxide dismutase but not by azide, an inhibitor of myeloperoxidase [20]. The results indicate that pravastatin inhibited $O_2^{\cdot -}$ generation in neutrophils stimulated by fMLF.

Effect of Pravastatin on TNF-α Primed and fMLF-Induced Tyrosine Phosphorylation

The effect of pravastatin on tyrosine phosphorylation of a 115-kDa protein in primed and stimulated PMN was investigated. fMLF-induced $O_2^{\cdot -}$ generation in PMN primed with TNF-α has been shown to be modulated through tyrosine phosphorylation of a 115-kDa protein [17, 18]. The experimental data show that pravastatin inhibited tyrosine phosphorylation of a 115-kDa protein (Fig. 6).

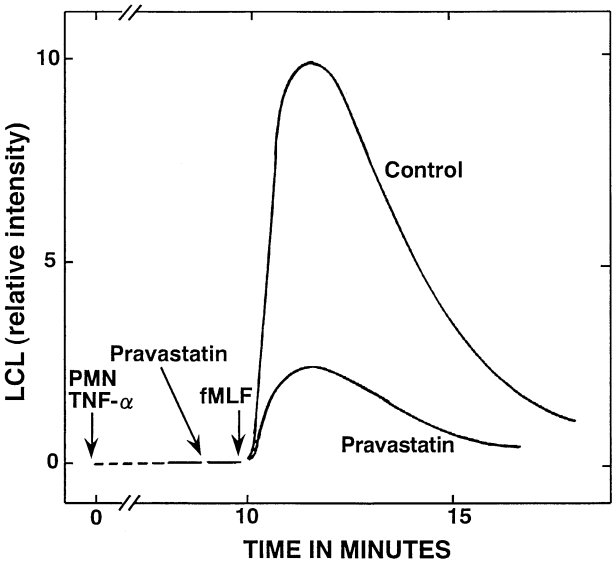

FIG. 5. Effect of pravastatin on the LCL of fMLF-stimulated PMN. The reaction mixture contained, in a final volume of 1 mL of KRP (pH 7.4), 1 mM glucose, 10 mM $CaCl_2$, 100 μM luminol, and 1 × 10^6 cells, at 37°. fMLF-induced LCL was determined in the presence or absence of 1 mM pravastatin. Final concentrations of TNF-α and fMLF were 20 U/mL and 25 nM, respectively.

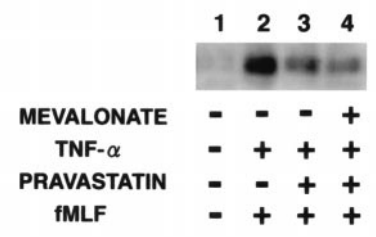

FIG. 6. Effect of pravastatin on tyrosine phosphorylation of a 115-kDa protein in PMN. PMN were preincubated with TNF-α in the presence or absence of mevalonate and/or pravastatin at 37° for 10 min, and then were treated with fMLF for 5 min. Proteins extracted from PMN (1×10^6 cells) were subjected to SDS–PAGE. Then they were transferred to PVDF membrane and stained with anti-phosphotyrosine antibody (PY-99) and peroxidase-conjugated anti-mouse IgG antibody as described in Materials and Methods. The concentrations of mevalonate, TNF-α, pravastatin, and fMLF were 200 μM, 20 U/mL, 1 mM, and 25 nM, respectively.

However, the inhibition was not reversed by mevalonate, in agreement with the experiments on fMLF-induced $O_2^{\cdot -}$ generation in neutrophils. These results indicate that pravastatin inhibited receptor-mediated $O_2^{\cdot -}$ generation in primed and stimulated PMN by decreasing tyrosine phosphorylation, but not by inhibiting cholesterol intermediate formation.

DISCUSSION

It is well known that fMLF-dependent $O_2^{\cdot -}$ generation in neutrophils is inhibited strongly by low concentrations of TK inhibitors [17, 18]. Several lines of evidence illustrate the functional importance of stimulated tyrosine phosphorylation in neutrophils [23–25]. One common feature of the responses to various agonists is the tyrosine phosphorylation of 115- to 120-kDa proteins [17, 18, 24, 25]. Recently, one of the 120-kDa proteins has been identified as the proto-oncogene product Cbl, a protein whose signaling role may be related to its combination of protein tyrosine phosphorylation sites [19, 26, 27]. However, it has been found, unexpectedly, that tyrosine phosphorylation of Cbl is not observed in neutrophils stimulated by fMLF [26]. It has been reported that the fMLF receptor belongs to the G-protein-coupled receptor superfamily [28] and that a G-protein-coupled receptor mediates the activation of PI3-K in neutrophils [29]. Furthermore, it has been found that tyrosine phosphorylation of PI3-K is inhibited by lovastatin, an inhibitor of HMG-CoA reductase [16]. Taken together, these data indicate that the signaling pathway of fMLF is associated with a G-protein-coupled receptor through tyrosine phosphorylation; therefore, the inhibition of the fMLF-induced respiratory burst by pravastatin probably occurs mainly through the inhibition of tyrosine phosphorylation activity.

Bokoch and Prossnitz [10] reported that treatment of DMSO-differentiated HL-60 cells with compactin, an inhibitor of HMG-CoA reductase, inhibits $O_2^{\cdot -}$ generation stimulated by fMLF or PMA. The inhibitory effects are prevented by the addition of exogenous mevalonate. These authors concluded that the inhibitory effects of the drug were due to the inhibition of the isoprenoid synthesis pathway. Furthermore, they postulated the existence of a protein that is required for the activity of NADPH oxidase and whose activity can be decreased by inhibitors of protein isoprenylation [10]. A recent study [30] also showed that treatment of neutrophils with low concentrations of pravastatin, such as 10–200 μM, significantly decreased chemotaxis triggered by fMLF. This effect was abolished in the presence of mevalonate. However, it also was reported that at none of the concentrations used (0.002 to 200 μM) did pravastatin affect neutrophil respiratory burst activity [30]. These results may indicate that the site of action of pravastatin in the case of chemotaxis is different from that involved in the respiratory burst of neutrophils.

We have shown that mevalonic acid did not prevent the inhibition of $O_2^{\cdot -}$ generation by pravastatin in short-term effects on the respiratory burst of neutrophils. It is probable that, as shown in Fig. 4A, a higher concentration of pravastatin was required for inhibition of the neutrophil respiratory burst in short-term experiments. The discrepancy between our results and those in other reports could be due to differences in the site of action of pravastatin. In addition, we found that pravastatin also inhibited tyrosine phosphorylation of a 115-kDa protein in the presence of mevalonate. These results suggest that the inhibitory effect of pravastatin is not connected with the mevalonate pathway.

In conclusion, our data indicate that pravastatin suppressed the $O_2^{\cdot -}$ generation in neutrophils stimulated by fMLF through inhibition of tyrosine phosphorylation, but not through inhibition of the mevalonate pathway.

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