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Impact of MK-886 on H_2O_2 Generation by Human Neutrophils and Cell Degranulation

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Abstract—Metabolic inhibitors can clearly affect different aspects of the functional activity of cells. This property was studied in the present work with respect to MK-886, a well-known inhibitor of the 5′-lipoxygenase-activating protein. It was found that this inhibitor in a micromolar concentration range (2-20 μM) induced in a dose-dependent manner H_2O_2 generation by human neutrophils and the release of lysozyme from the cells. The MK-886-induced activation of neutrophils was accompanied by a significant decrease in N-(1-pyrene)maleimide-accessible SH-groups in the cells. According to its activity, MK-886 can be considered an agonist that causes up-regulation of inherent neutrophil functions. In summary, the results indicate that during the application of MK-886 as a 5′-lipoxygenase inhibitor in neutrophils, the impact of the compound on the functional activity of the cells should be taken into consideration.

Key words: MK-886, neutrophil, lipoxygenase, hydrogen peroxide, degranulation, lysozyme

The application to cell systems of metabolic inhibitors selectively affecting isolated enzymes clearly raises the question of the actual targets of the drugs at the level of multiple complexes of interacting enzymatic and structural cellular elements. This problem is very important with respect to lipoxygenase inhibitors that have appeared to exhibit ambivalent actions on various aspects of the functional activity of neutrophils, namely the generation of reactive oxygen species, degranulation, chemotaxis, and apoptosis [1-6]. In particular, the argued suggestion that the impairment of neutrophil functions by the respective compounds cannot be attributed only to the inhibition of the lipoxygenase pathway was put forward by Ozaki and coworkers in 1986 [1]. Subsequent investigations have shown that lipoxygenase inhibitors are able not only to inhibit but also to activate different cell functions as well as to serve as priming agents with respect to the NADPH oxidase system of neutrophils. For example, this aspect was revealed for such substances as 3-[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]thio]-N-methyl-N-[2-(2-pyridinyl)ethyl]propanamide (SC-41661A) [2] and WY 50,295 [7], which enhanced superoxide generation by human neutrophils and HL-60 cells induced by N-formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol-12-myristate-13-acetate (PMA), respectively.

In our previous studies, we detected a direct stimulating, not only a priming, effect of the lipoxygenase inhibitor MK-886 on H_2O_2-generating activity of human neutrophils [8, 9]. Recently, a similar effect of MK-886 has been independently reported [10] with the example of analysis of oxidizing reactions (hydrolysis of carboxydichlorofluorescein diacetate) in FL5.12 cells (an IL-3-dependent murine prolymphoid progenitor cell line). It should be noted that, in contrast to other lipoxygenase inhibitors, the molecular mechanism of the inhibiting activity of MK-886 is determined by its interaction not with lipoxygenase but with 5′-lipoxygenase activating protein (FLAP) [11]. This point seems to be very interesting because FLAP-mediated bifurcation of cellular responses through various signaling pathways is not excluded in this case. Apparently, the applied concentration of MK-886 has key importance for realization of the

Abbreviations: DPI) diphenyleneiodonium; FLAP) 5′-lipoxygenase activating protein; FMLP) N-formyl-methionyl-leucyl-phenylalanine; NDGA) nordihydroguaiaretic acid; NEM) N-ethylmaleimide; MK-886) 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid; PBS) phosphate buffered saline; PMA) phorbol-12-myristate-13-acetate; TFP) trifluoperazine; VAA) galactoside-specific *Viscum album* L. agglutinin.

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respective cellular responses since the inhibition of lipoxygenase activity was observed in a nanomolar range of doses [12] while stimulation of an increase in cytosolic Ca^{2+} and apoptosis required a micromolar dosage [6, 13].

The purpose of the present work was to study in detail effects of MK-886 on inherent reactivity of human neutrophils and to determine the concentration range that is crucial for this compound to activate plasma membrane NADPH oxidase of cells and lysozyme release from intracellular granules.

MATERIALS AND METHODS

Preparation of neutrophil suspension. Human neutrophils were isolated from group O(I) donor blood stabilized in glugicirum by centrifugation through a Histopaque-1077 density gradient as described elsewhere [14]. The cells were suspended in phosphate-buffered saline (PBS) containing 10 mM Na_2HPO_4/KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.35) and stored at 4°C. Cell viability, which was determined using trypan blue staining, always exceeded 96%.

Measurements of H_2O_2 generation by human neutrophils. H_2O_2 generation by cells was monitored by the scopoletin fluorescent method described earlier [15]. MK-886 was added at a tested concentration to 1.5 ml of neutrophil suspension ($2 \cdot 10^6$ cells/ml in PBS, 37°C) directly in a fluorimeter cuvette containing 1 μM scopoletin, horseradish peroxidase (20 μg/ml), and 1 mM NaN_3. The kinetics of scopoletin oxidation was recorded as the decrease in fluorescence intensity of samples at 460 nm (excitation at 350 nm). The rate of H_2O_2 generation was determined as a tangent of the maximal slope of the lineal part of the kinetic curve. Fluorescent measurements were performed using a LSF 1211A computerized spectrofluorimeter from SOLAR (Minsk, Belarus).

Analysis of neutrophil degranulation. Degranulation of neutrophils was assessed as the release of lysozyme into the extracellular medium according to the previously described method [16]. Cell suspensions ($3 \cdot 10^6$ cells/ml in PBS) were incubated for 20 min at 37°C with different concentrations of MK-886 in polypropylene Eppendorf tubes. The analysis of neutrophil degranulation was carried out in the absence of cytochalasin B, i.e., in conditions providing the release of enzymes mainly from specific (secondary) granules [14, 16]. To stop the degranulation response, the samples were placed on ice and centrifuged for 15 min at 3000 rpm in an OPN-3 centrifuge (Russia). To measure lysozyme activity, 200 μl of the supernatant was added to 1.8 ml of a suspension of lyophilized *Micrococcus lysodeikticus* cells (0.2 μg/ml) in 0.1 M Na_2HPO_4/KH_2PO_4 buffer, pH 6.2 at 25°C, and increase in light transmission at 450 nm was recorded for 4 min using a KFK-2 colorimeter (Russia). The amount of lysozyme released from the neutrophils was calculated as the percentage of total enzyme activity in cells treated with 0.1% Triton X-100.

Identification of free SH-groups in cells. The fluorescent indicator of thiols N-(1-pyrene)maleimide was used to assess the total level of SH-groups in human neutrophils as described elsewhere [17]. Cells (10^6 cells/ml) were incubated for 5 min at 37°C with different concentrations of MK-886 and then N-(1-pyrene)maleimide was added to reach a final concentration of 1 μM. Kinetics of any increase in fluorescence intensity at 377 nm (excitation at 344 nm) due to binding of the probe to free sulfhydryl groups was monitored using the LSF 1211A spectrofluorimeter. The stationary level of N-(1-pyrene)maleimide fluorescence achieved during 10 min was considered as the indicator of SH-groups in the cells.

Reagents. Nordihydroguaiaretic acid (NDGA), trifluoperazine (TFP), dimethylsulfoxide, horseradish peroxidase (type II), scopoletin, catalase from bovine liver, Histopaque-1077, N-ethylmaleimide (NEM), FMLP, lyophilized *Micrococcus lysodeikticus* bacterial cells, and Triton X-100 were purchased from Sigma (Germany); sodium azide was from Fluka (Germany); 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid sodium salt (MK-886) and diphenyleneiodonium chloride (DPI) were from Calbiochem (Germany); N-(1-pyrene)maleimide was from Molecular Probes (The Netherlands).

RESULTS

The basal level of H_2O_2 generation by neutrophils in the absence of agonists was negligible, and the level of scopoletin fluorescence remained practically unchanged during 15-30 min. The addition of MK-886 to a suspension of neutrophils kept at 37°C led to a dose-dependent oxidation of scopoletin (Fig. 1). The kinetics were similar to those recorded in the analysis of H_2O_2 generation by neutrophils stimulated by such agonists of plasma membrane NADPH oxidase as FMLP, digitonin, and galactoside-specific mistletoe lectin (*Viscum album* L. agglutinin, VAA) [18]. It should be noted MK-886 failed to affect scopoletin fluorescence in cell-free media, excluding a nonspecific interaction of the compound with components of the reaction mixture.

To confirm the fact of MK-886-induced H_2O_2 generation by neutrophils due to the activation of the plasma membrane NADPH oxidase system, the effects of NADPH oxidase inhibitors on the detected response were studied. It was found that the addition of MK-886 (6 μM) to neutrophils in the presence of catalase (400 μg/ml or 1000 U/ml), NEM (10 μM), TFP (10 μM), or DPI (10 μM) did not induce any changes in scopoletin fluorescence. These data suggest that MK-886 up-regulates assembly of functionally active NADPH oxidase complex in the plas-

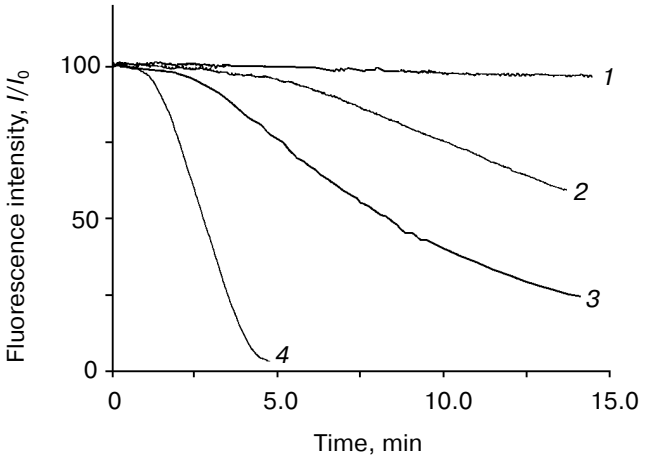

Fig. 1. Effect of different doses of MK-886 on H_2O_2 generation by human neutrophils ($2 \cdot 10^6$ cells/ml): *1*) control (without MK-886); *2*) 3 μM; *3*) 4.5 μM; *4*) 6 μM. The measurements were performed at 37°C. The suspension of neutrophils contained 1 μM scopoletin, horseradish peroxidase (20 μg/ml), and 1 mM NaN_3. Intensity of scopoletin fluorescence was measured 460 nm (excitation at 350 nm).

ma membrane of neutrophils. The stimulatory effect of MK-886 with regard to H_2O_2 generation significantly depends on the agonist concentration in the range of 2-20 μM (Fig. 2). The dose–response curve covering a rather narrow range of concentrations has a typical sigmoid shape that indicates the functional specificity of binding sites of MK-886 in cells and/or its equilibrium binding to various molecular targets.

Additionally to the activation of the NADPH oxidase system of neutrophils, MK-886 induced cellular degranulation, i.e., release of lysozyme into the extracellular media. It was found that supernatants of neutrophils ($3 \cdot 10^6$ cells/ml) treated with various concentrations of MK-886 for 20 min at 37°C exhibited a lysing activity with respect to *Micrococcus lysodeikticus* bacterial cells. The respective dose–response curve was practically identical to those describing generation of H_2O_2 (Fig. 2). The tested doses of MK-886 failed to affect lysozyme activity in a cell suspension treated with Triton X-100 (0.1%), this excluding possible interfering effects of the inhibitor on lysis of *Micrococcus lysodeikticus* cells.

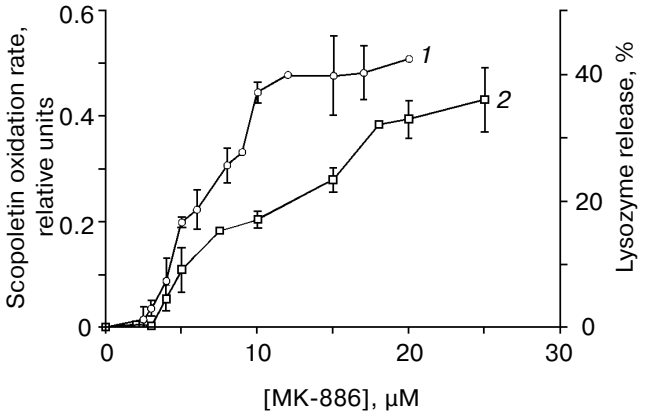

Fig. 2. Dose–response curves for MK-886 in human neutrophils: *1*) generation of H_2O_2; *2*) release of lysozyme. The conditions of measurements are presented in "Materials and Methods". The vertical bars correspond to standard deviations of three independent experiments with cells from different donors.

The capacity of MK-886 to stimulate H_2O_2 generation and lysozyme release in human neutrophils shows that this compound is an agonist causing the up-regulation of neutrophil reactivity like such agents as FMLP, PMA, VAA, and the Ca^{2+}-ionophore A23187 [14, 19]. To compare the potential activity of MK-886 with such agonists, FMLP- and VAA-induced H_2O_2 generation by human neutrophils was studied in accordance with the earlier described protocol [18]. It was found that all three compounds elicited similar effects, i.e., the rates of scopoletin oxidation induced by MK-886 (10 μM), FMLP (20 nM), and VAA (2.5 μg/ml) were 0.448, 0.695, and 0.356 arbitrary unit, respectively.

The treatment of neutrophils (10^6 cells/ml) with different doses of MK-886 for 5 min at 37°C was also accompanied by changing of cellular redox status evaluated as the level of SH-groups in cells accessible to N-(1-pyrene)maleimide. Under these conditions, the number of thiol groups was reduced in a dose-dependent manner reaching almost 50% level of reduction in the presence of 10 μM MK-886 (Fig. 3).

DISCUSSION

The results demonstrate a new property of MK-886 (a FLAP inhibitor)—the ability to stimulate plasma membrane NADPH-oxidase activity and degranulation of human neutrophils. This activity of MK-886 is detected in the concentration range of 2-20 μM that is far above the doses inhibiting 5′-lipoxygenase activity in cells [12].

The molecular mechanisms of the action of MK-886 on neutrophils can be associated with several variants of formation of the respective signaling cascade. First, the assembly of active NADPH oxidase complex in many cases is a consequence of ligand–receptor interaction triggering a well-known chain of signaling reactions (ligand → receptor → G-proteins → formation of secondary messengers → mobilization of intracellular Ca^{2+} and activation of protein kinases → functional response) [20]. In particular, the receptor mechanism leading to activation of neutrophils is realized for FMLP and lectins, which have specific binding sites on the cell surface [21, 22]. To date, it is not known whether there are MK-886 receptors on the surface of neutrophils; it is possible only to note that FLAP is an integral protein and is localized mainly in

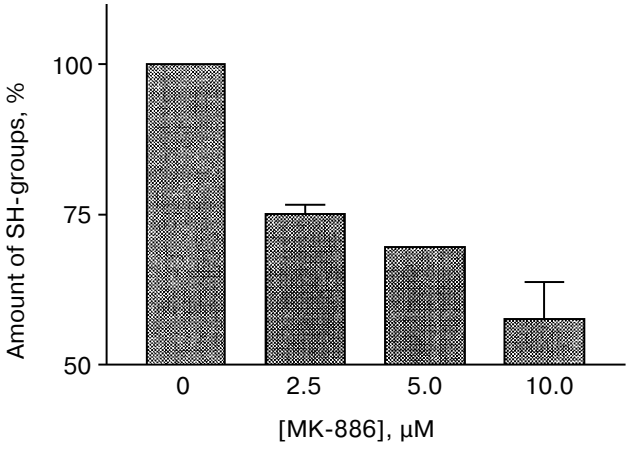

Fig. 3. Decrease in SH-groups in human neutrophils (10^6 cells/ml) exposed for 3 min at 37°C to different doses of MK-886. The stationary level of fluorescence of 1 μM N-(1-pyrene)maleimide, a sulfhydryl reagent, in a suspension of untreated neutrophils was taken as 100%. The vertical bars correspond to standard deviations of three independent experiments with cells from different donors.

nuclear membrane of cells and, particularly, in endoplasmic reticulum [23].

Second, MK-886 is soluble in DMSO and due to its amphiphilic properties can easily penetrate through the plasma membrane of cells. In this connection, a comparison arises with activation of neutrophils by PMA, which directly activates intracellular protein kinase C without the participation of membrane receptors [24]. Since the phosphorylation of cytoplasmic components of NADPH oxidase is a critical step for assembly of the active complex, an influence of MK-886 on this process would have paramount importance.

The effects of MK-886 can be secondary and mediated by its interaction with FLAP or other regulatory target proteins in cells resulting in the subsequent activation parts of the intracellular signaling reactions that are involved in the processes of translocation of components the NADPH oxidase system to the plasma membrane. This idea is supported by our results indicating that H_2O_2 generation is associated with MK-886-induced release of lysozyme from secondary granules proceeding in the absence of cytochalasin B. The same granules contain a significant part of cytochrome b_{558}, and its translocation to the plasma membrane is an important element of intracellular events leading to priming of neutrophils [25]. Among cellular effects of MK-886 that can affect assembly of catalytically active NADPH oxidase complex, the increase in intracellular Ca^{2+} [26] and Ca^{2+} flows [27] should also noted.

The secondary effects of MK-886 can hardly be associated with inhibition of 5′-lipoxygenase as most of its other inhibitors suppress the activity of neutrophils [1, 3, 4]. In this respect, our finding is remarkable that the treatment of neutrophils with MK-886 resulted in a decrease in the amount of cellular sulfhydryl groups, apparently due to the H_2O_2-mediated auto-oxidation of intracellular thiols as occurs during the reduction of menadione by hepatocytes [28], cardiomyocytes [29], and rat thymocytes [30]. Otherwise, it would be possible to expect an augmentation of glutathione-controlled synthesis of leukotriene B_4 [31], a receptor agonist of neutrophils, which is excluded because of the presence MK-886 in the cellular suspension.

The presence in cells of other targets of MK-886 than FLAP has the important significance of disclosing molecular mechanisms of neutrophil activation induced by this compound. A FLAP-independent pathway of MK-886-mediated apoptosis was demonstrated recently with respect to lymphocytic cells WSU and FL5.12 [10]. Fatty acid-binding proteins participating in intracellular signaling processes [32, 33] should be considered as the most suitable candidates to bind MK-886. Because the amino acid composition of FLAP is known [11], the search for homologous sequences among the known regulatory proteins using modern computer technologies and Internet seems to be also very promising. In conclusion, it is important to note that the activating effect of MK-886 on the functioning of plasma membrane NADPH oxidase and degranulation of neutrophils should be taken into account for screening of lipoxygenase inhibitors as potential antitumor drugs [34].

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