Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 38-46

THE DIACYLGLYCEROL KINASE INHIBITOR R59022 POTENTIATES SUPEROXIDE PRODUCTION BUT NOT SECRETION INDUCED BY fMET-LEU-PHE: EFFECTS OF LEUPEPTIN AND THE PROTEIN KINASE C INHIBITOR H-7

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Received August 24, 1987

SUMMARY: The addition of low concentrations of the chemotactic factor fMet-Leu-Phe to rabbit neutrophils in the absence of cytochalasin B produces very little superoxide. This level of superoxide can be greatly increased in neutrophils pretreated for 30 min with 10 μM of the diacylglycerol kinase inhibitor R59022. This potentiation occurs also in the presence of cytochalasin B. In addition, while the small level of superoxide generated by fMet-Leu-Phe is not inhibited by the protein kinase C inhibitor 1-(5-isoquinoline-sulfonyl)-2-methyl piperazine (H-7), the increase by R59022 is completely abolished by this compound. In addition, this increase can be potentiated further by leupeptin. Unlike superoxide generation, the release of lysozyme or N-acetyl-β-glucosaminidase produced by fMet-Leu-Phe is not stimulated by R59022. The results presented here suggest that stimulation of the oxidative burst requires the generation and the maintenance of a sufficient amount of diacylglycerol and/or the rearrangement of the cytoskeleton such as the inhibition of actin polymerization. Furthermore, the membrane-associated form of protein kinase C is the one responsible for the activation of the oxidative burst. The relationship between protein kinase C activation and the stimulated oxidative burst and the physiological role of chemotactic factors in the functions of the neutrophils are discussed. © 1987 Academic Press, Inc.

The addition of the chemotactic factor fMet-Leu-Phe to neutrophils causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the generation of 1,2 diacylglycerol (DG) and the inositol 1,4,5 trisphosphate (IP_3) (1-14). Both of these products are thought to act as second messengers, mediating one or more neutrophil response (1-14). DG activates protein kinase C (15), which is thought to be involved in stimulated superoxide generation and possibly degranulation. IP_3 mobilizes calcium from non-mitochondrial stores (16). The rise in intracellular concentration of calcium, either directly and/or through the calmodulin, is thought to be involved in several neutrophil functions.

0006-291X/87 $1.50
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Although the evidence strongly suggests that activation of protein kinase C leads to the generation of superoxide, it is not yet clear if the chemotactic factor fMet-Leu-Phe stimulates O_2^- release through a pathway that uses PKC, independently of PKC or other kinases. This is particularly relevant when no cytochalasin B is used. There are two experimental observations which are inconsistent with a role for protein kinase C in superoxide generation produced by fMet-Leu-Phe. First, protein kinase inhibitors such as H-7 and C-I do not inhibit O_2^- production produced by fMet-Leu-Phe (11,17,18). On the other hand, these inhibitors reduce PMA-induced O_2^- production (11,17). Second, unlike PMA, fMet-Leu-Phe does not cause a translocation of cytosolic protein kinase C to a particulate fraction (19,20).

The amount of diacylglycerol produced following stimulation of neutrophils with fMet-Leu-Phe in the absence of cytochalasin B is small and short lived (21). In addition, fMet-Leu-Phe induced phosphorylation of certain proteins which are thought to be phosphorylated by protein kinase C activation is small and very transient (22). The transient nature of the formed DG is due to its conversion to phosphatidic acid through the action of DG kinase and possibly to arachidonic acid through the action of diacylglycerol lipase (23). This transient nature of diacylglycerol production may account for this apparent lack of involvement of PKC in superoxide generation produced by fMet-Leu-Phe.

The present studies were undertaken to examine the effects of fMet-Leu-Phe on superoxide generation and degranulation in control, diacylglycerol kinase inhibitor R59022, protein kinase C inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H-7) and R59022 plus H-7 treated rabbit neutrophils.

MATERIALS AND METHODS

Rabbit peritoneal neutrophils collected from 4 or 16 hour exudates were washed and resuspended in Hanks' balanced salt solution buffered with 10 mM Hepes (7.3) as previously described (24). The buffered solution contained no magnesium or protein, and the cells were suspended at 10^7 cells/ml. Cytochalasin B (2 µg/ml) was used only when indicated. The diacylglycerol kinase inhibitor R59022 was used at a concentration

of 10 μM, and the cells were incubated with this concentration for 30 min. The protein kinase C inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, H-7, was used at 25 μM, and the cells were incubated with this compound for 5 min.

Degranulation was measured as previously described (24). Briefly, enzyme release was measured after 5 minutes incubation (37°C) with fMet-Leu-Phe in the presence of 2 μg/ml cytochalasin B, and the cells were centrifuged in the cold (400 x g, 3 min). The enzymes measured were lysozyme and N-acetyl-β-glucosaminidase. Lactate dehydrogenase release, a marker of cell damage, did not differ in control, untreated cells, or in the experimentally manipulated cells and in any case did not exceed 6% of the total cell content.

Superoxide production was determined at 37°C using the method of Cohen and Chovaniec (25). The release of O_2^- was determined as the change in absorbance at 550 nm from baseline using a Beckman spectrophotometer Model DU-50. The reaction mixture contains 2.5 x 10^6 cells, cytochrome c (238 μM), sodium azide (2 mM), $CaCl_2$ (2 mM) and $MgCl_2$ (2.4 mM). The reaction was carried out for 2 minutes. At the end of the specified time the reaction was stopped by the addition of NEM (0.5 mM). The results reported here refer to endpoint measurements. Generation of O_2^- was calculated by subtracting the absorbance change in the presence of superoxide dismutase, SOD, (2 mM) from that in its absence and then dividing by the value of 21.1 x 10^3 M^{-1} cm^{-1} for the molar extinction coefficient.

Formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) was purchased from Peninsula Labs. (San Carlos, CA), cytochalasin B from Aldrich Chem. Co. (Milwaukee, WI), 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine (H-7) from Seikagaku America, Inc. (St. Petersburg, FL), and the diacylglycerol kinase inhibitor R59022 from Janssen Life Science Section (New Jersey).

RESULTS AND DISCUSSION

Generation of superoxide in control and the diacylglycerol kinase inhibitor R59022-treated rabbit neutrophils stimulated by various concentrations of fMet-Leu-Phe has been measured, and the results are shown in Figure 1. Note that the dose-response curve is shifted to the left in the presence of the inhibitor. In another set of experiments, the effect of the protein kinase C inhibitor, H-7, on the potentiation by R59022 of the fMet-Leu-Phe stimulated superoxide production was also examined and the results are summarized in Table 1 and Figure 2. It is interesting to point out that while the superoxide production produced by fMet-Leu-Phe is not affected by H-7, the potentiation produced by R59022 is completely abolished by H-7.

Protein kinase C accounts for the majority of the kinase activities of the neutrophils, and its intracellular distribution is to some extent

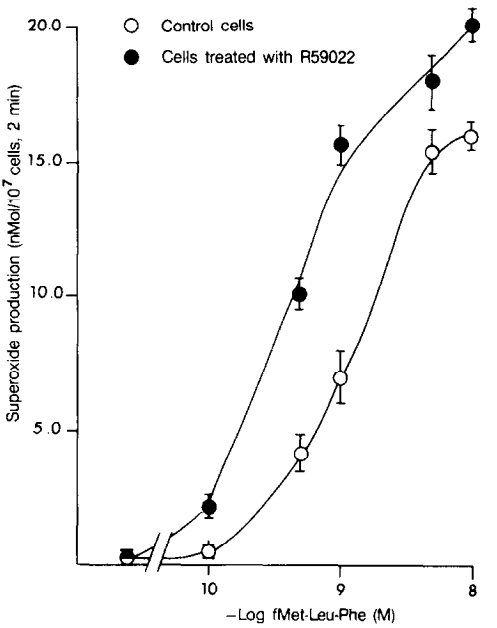


Figure 1. Effect of various concentrations of the chemotactic factor-fMet-Leu-Phe on superoxide production in control and R59022 treated rabbit neutrophils. The cells were incubated with 10 μM R59022 for 30 min. Stimulation with fMet-Leu-Phe was carried out for 2 min. Each point represents the mean ± SEM of at least three separate experiments.

dependent on the state and nature of the activation of the cell. Following stimulation, the cytosolic protein kinase C, and a calcium activated

TABLE 1

Effect of the protein kinase C inhibitor 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, H-7,on the potentiation by the diacylglycerol inhibitor R59022 of fMet-Leu-Phe induced superoxide production

Exp. Condition	Superoxide Production ($nMol/10^7$ cells, 2 min)**
Control Cells	6.5 ± 1.0
R59022 treated cells*	18.5 ± 1.2
H-7 treated cells*	7.9 ± 0.8
R59022 followed by H-7*	8.5 ± 1.4

*Cells were treated with R59022 (10 μM) for 30 minutes and with H-7 (25 μM) for 5 minutes.

**The cells were stimulated with fMet-Leu-Phe (1 nM) for two minutes. Each value represents the mean ± SEM of at least three separate experiments. The basal level of superoxide production is less than 1 $nMol/10^7$ cells/2 min. R59022 and H-7 had no effect on this basal value.

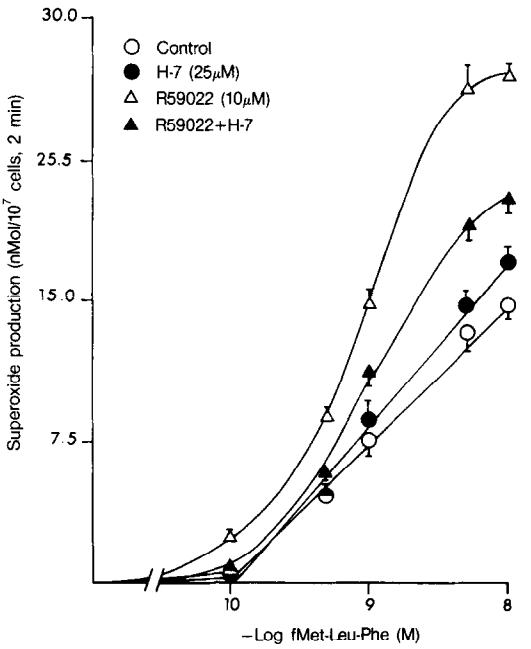


Figure 2. Superoxide production produced by various concentrations of fMet-Leu-Phe in control, H-7, R59022 and R59022 + H-7 treated cells. The cells were incubated with R59022 (10 μM) for 30 min and with H-7 (25 μM) for 5 min. Stimulation with fMet-Leu-Phe was carried out for 2min. Each point represents the mean ± SEM of at least three separate experiments.

neutral proteinase, CANP, are translocated to the plasma membrane where active CANP promotes proteolytic conversion of PKC (13,14,15,19,20,26). Leupeptin is thought to inhibit the neutral proteinase activity (13,26). In order to examine further the role of PKC, the effect of leupeptin on superoxide generation produced by fMet-Leu-Phe in control and R59022-treated cells was tested. The results, summarized in Table 2, clearly show that leupeptin potentiates the stimulated oxidative burst in R59022-treated neutrophils. In the presence of cytochalasin B, the level of superoxide production is much higher than in its absence, and the inhibitor R59022 increases this level further. Furthermore, leupeptin potentiates the generation of superoxide produced by fMet-Leu-Phe in the presence of cytochalasin B in R59022-treated cells.

The effect of the inhibitor R59022 on lysozyme and N-acetyl-β-glucosaminidase release produced by various concentrations of fMet-Leu-Phe in the presence of 2 μg/ml of cytochalasin B has been studied.

TABLE 2

Effects of leupeptin and cytochalasin B on the potentiation by the diacylglycerol inhibitor R59022 of fMet-Leu-Phe induced superoxide production

Exp. Condition	Superoxide Production ($nMol/10^7$ cells, 2 min)**	
	- Cytochalasin B	+ Cytochalasin B
Control Cells	5.0 ± 1.5	46.0 ± 4.5
R59022 treated cells*	19.2 ± 3.0	56.0 ± 5.3
Leupeptin treated cells*	3.0 ± 0.8	41.8 ± 6.2
R59022 followed by Leupeptin*	28.0 ± 2.5	66.0 ± 4.0

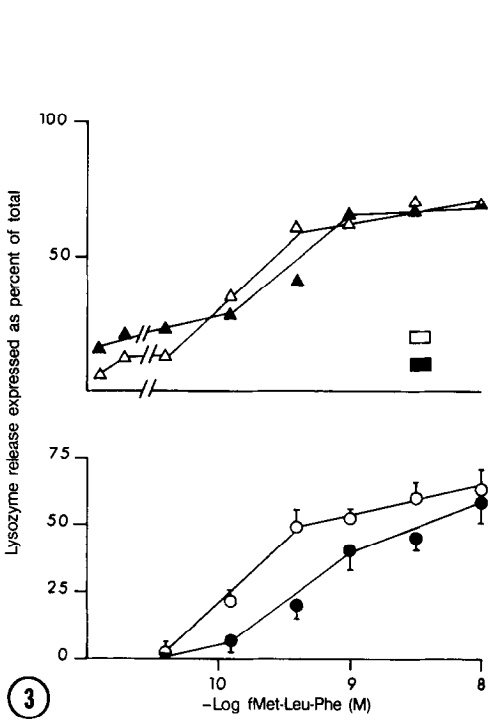
*Cells were treated with R59022 (10 μM) for 30 minutes and with leupeptin (100 μM) for 5 minutes.

**The cells were stimulated with fMet-Leu-Phe (1 nM) for two minutes. Each value represents the mean ± SEM of at least three separate experiments. Cytochalasin B concentration was 2 μg/ml. The basal level of superoxide production is less than 1 $nMol/10^7$ cells, 2 min. This level was not affected by leupeptin or R59022.

The results are shown in Figures 3 and 4. Unlike superoxide generation, fMet-Leu-Phe-induced degranulation is not significantly enhanced in neutrophils treated with 10 μM R59022. In the absence of fMet-Leu-Phe, the inhibitor R59022 produces an increase in the level of degranulation. If this enhancement is subtracted from the values of degranulation produced by fMet-Leu-Phe, a small but significant inhibition is observed in cells treated with R59022, particularly at low concentrations of fMet-Leu-Phe. Note that R59022 does not potentiate the small lysozyme release produced by fMet-Leu-Phe in the absence of cytochalasin B (Figure 3, □ , ■).

The results presented above make five separate though interrelated points.

1. Inhibition of the conversion of diacylglycerol to phosphatidic acid by R59022 causes a significant increase in superoxide production produced by the chemotactic factor fMet-Leu-Phe. This increase is



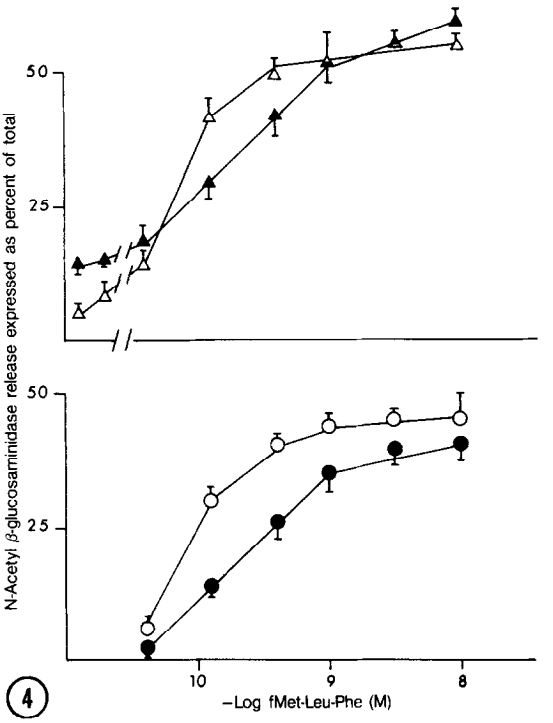


Figure 3. Lysozyme release produced by various concentrations of fMet-Leu-Phe in control and R59022-treated neutrophils. The symbols are as follows: △, control cells stimulated in the presence of 2 µg/ml cytochalasin B; ▲, cells treated with R59022 (10 µM) for 30 min and stimulated in the presence of cytochalasin B; □, control cells stimulated without cytochalasin B; ■, R59022-treated cells stimulated in the absence of cytochalasin B; ○, control cells in which the release produced by cytochalasin B alone without the addition of fMet-Leu-Phe is subtracted from each point, and ●, R59022 treated cells in which the release produced by cytochalasin B alone is subtracted from each point. The data are taken from a representative experiment.

Figure 4. N-acetyl β-glucosaminidase release produced by various concentrations of fMet-Leu-Phe. Conditions are the same as in Figure 3.

blocked by the protein kinase C inhibitor H-7 suggesting that it is mediated through protein kinase C activation. It is worth pointing out that sphinganine, a postulated protein kinase C inhibitor, at least in our hands, is cytotoxic, and it inhibits many cell responses including the rise in intracellular concentration of free calcium (unpublished data, 27).

2. Leupeptin potentiates the increase in the fMet-Leu-Phe induced superoxide generation in R59022-treated cells. This suggests strongly that it is the membrane-associated form of protein kinase C which is responsible for the stimulation of NADPH oxidase.

3. The small level of superoxide production, if any, by chemotactic factors, is most likely not mediated through the activation of protein kinase C. The increased oxidative burst activity at the site of infection is probably due, in part, to the activation of protein kinase C by the diacylglycerol generated in response to phagocytosis.

4. The increase in the presence of cytochalasin B is most likely due to the rearrangement of cytoskeleton which includes the inhibition of actin polymerization.

5. R59022 does not potentiate lysozyme release regardless of whether cytochalasin B is present or not. This suggests that activation of protein kinase C may not be involved in the secretion by these cells.

These findings suggest that under physiological conditions (low concentrations and no cytochalasin B), the main physiological function of chemotactic factors such as fMet-Leu-Phe is to cause cell movements and not to generate significant superoxide or degranulation. It would not be physiologically beneficial if the neutrophils generate superoxide or degranulate in response to the chemotactic agent while migrating toward the site of infection. The inability of the chemotactic factors to produce superoxide is most likely due to the small magnitude and the transient nature of the diacylglycerol produced by these agents. Accordingly, a significant amount of superoxide generation requires the production and maintenance of a sufficient amount of diacylglycerol and/or cytoskeletal rearrangement. When there is a sufficient amount of diacylglycerol generated, some of the cytosolic protein kinase C is translocated to the membrane, and this membrane-associated form of the protein kinase C is the one responsible for the activation of the oxidative burst. Physiologically, these changes occur most likely at the site of infection.

ACKNOWLEDGMENT

This work is supported in part by NIH grant GM-37694-10 and AI-09648.

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