Activation by Saturated and Monounsaturated Fatty Acids of the $\text{O}_2$-generating System in a Cell-free Preparation from Neutrophils*

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Saturated and monounsaturated fatty acids with appropriate chain length such as laurate and oleate activated an $\text{O}_2$-generating enzyme system in a cell-free preparation from porcine neutrophils. The activated preparation catalyzed a stoichiometric conversion of $\text{O}_2$ to $\text{O}_2^-$ by utilizing NADPH as the electron donor. The preparation contained both membrane and soluble fractions and, upon separation into subfractions, the $\text{O}_2$-generating activity resided exclusively in the membrane fraction. Polyunsaturated fatty acids including arachidonate also activated the system, but they concurrently stimulated NADPH-independent $\text{O}_2$-consumption reactions which yield neither $\text{O}_2^-$ nor $\text{H}_2\text{O}_2$. The amount of such a non-$\text{O}_2^-$-producing $\text{O}_2$ consumption often reached twice as much as that of $\text{O}_2^-$ production.

For the activation of the $\text{O}_2^-$-generating system in the membrane, the presence of the soluble fraction was essential. However, the soluble fraction was no longer effective when once used for the activation, suggesting that the effective component(s) in the fraction was consumed or translocated to the membrane during the activation. When the activated membrane was incubated with delipidated albumin, the activity was lost with concomitant decreases in the amount of membrane-associated fatty acids. The lost activity was restored by the replenishment of the fatty acid in the presence of a fresh soluble fraction. We also found that Ca$^{2+}$ augmented a non-$\text{O}_2^-$-producing $\text{O}_2$ consumption in the cell-free preparation by unsaturated fatty acids and interfered with the activation of the $\text{O}_2^-$-generating system, especially that by saturated fatty acids.

The abbreviations used are: $\text{O}_2^-$, superoxide anion; HBSS, HEPES-buffered Hank's balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate; EGTA, ethylenebis(oxyethylenenitri1o)tetraacetic acid.

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*cis-Unsaturated fatty acids such as arachidonate and oleate have been known to stimulate the release of superoxide anion ($\text{O}_2^-$) from intact neutrophils and macrophages (1–4). The stimulation was reversed by the removal of fatty acids from the cells with delipidated albumin and was restored by the replenishment of the fatty acids (2). It was also known that the stimulation was accompanied by morphological changes of the cells (2). Saturated fatty acids such as myristate and palmitate and trans-unsaturated fatty acids including linoleic-laidate were described as inert both in stimulating the $\text{O}_2^-$ release and in provoking the morphological changes. The effects of fatty acids were thus interpreted to be due to the perturbation of plasma membrane caused by the intercalated fatty acids with the double bond(s) in the cis-configuration (1, 2). Recently, an NADPH-dependent $\text{O}_2^-$-generating system in cell-free preparations from neutrophils or macrophages was shown to be activated by unsaturated fatty acids (5–10) and sodium dodecyl sulfate (11), but again not by saturated fatty acids such as stearate and palmitate (6, 9, 10).

In contrast to above observations, Kakinuma and her associates (12–15) have repeatedly reported that saturated fatty acids with appropriate chain length were also good stimuli for the $\text{O}_2^-$-generating system in intact neutrophils from various sources. Recently, we were also able to show that, besides unsaturated fatty acids, saturated fatty acids were effective in stimulating the $\text{O}_2^-$ release in human and porcine neutrophils (16). In these papers, evidence was provided that the discrepancy results on the effects of saturated fatty acids are due at least in part to the different Ca$^{2+}$ concentrations in the medium employed in each study. It has been known that Ca$^{2+}$ interacts with fatty acids affecting their solubilities (17) or altering the physicochemical states of fatty acids which were inserted in the biological membrane (18).

In the present study, we examined the activation of the $\text{O}_2^-$-generating system in a cell-free preparation from porcine neutrophils by various fatty acids with special emphasis on the stoichiometry between $\text{O}_2$ generation and $\text{O}_2$ consumption in the activated reaction. The stoichiometry was determined by using a heme-substituted horseradish peroxidase as a trapping reagent for both $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ (19). The results show that all kinds of straight-chain fatty acids with appropriate carbon numbers (C$_{18}$–C$_{20}$) are capable of activating the $\text{O}_2^-$-generating system in the cell-free preparation irrespective of their structures. The reported inertness of saturated and trans-monounsaturated fatty acids in the activation of $\text{O}_2^-$-generating system was presumably due to the way of adding them to the reaction mixture. A difference was noted among the effects of various kinds of fatty acids. The activation by...
saturated and monounsaturated fatty acids was specific to the O₂-generating enzyme system in the sense that they did not stimulate other O₂-consuming reactions, while polyunsaturated fatty acids stimulated both O₂-generating and non-O₂-generating O₂ consumptions. We also found that the O₂-generating system in the membrane fraction could be activated and deactivated in ultraviolet light, and the activity was preserved. The presence of a highly sensitive membrane was not necessary to maintain the activity. The significance of these results is discussed. A preliminary report pertinent to a portion of this work has appeared (16).

MATERIALS AND METHODS

Isolation of Neutrophils—Porcine neutrophils were isolated as described previously (20) with minor modifications. The whole blood (1 liter) was anticoagulated with heparin with mixed with an equal volume of phosphate-buffered saline at pH 7.4, followed by sedimentation of erythrocytes in 0.83% dextran T-500 (Pharmacia LKB Biotechnology Inc.) at 1 × g, 25 °C. The granulocyte-rich supernatant was collected and centrifuged at 100 × g for 10 min, 0°C. The supernatant was further centrifuged at 280,000 × g for 10 min, and the pellet (containing 88-97% neutrophils, and the yield was 2-10 × 10⁷ cells/ml) was quickly thawed in a water bath at 39 °C with vigorous stirring. The cells were found to be disrupted upon ultrasonic oscillation at 0°C for 30 s. The presence of a highly sensitive membrane was always required for the activation, but it was not necessary to maintain the activity. The significance of these results is discussed. A preliminary report pertinent to a portion of this work has appeared (16).

Activation of Neutrophil O₂-generating System by Fatty Acids

Activation of Neutrophil O₂-generating System by Fatty Acids—

The generation of both O₂ and H₂O₂ generation and O₂ consumption—

The generation of both O₂ and H₂O₂ were determined spectrophotometrically by using diacyltyrodehydrol-substituted horseradish peroxidase as the trapping reagent (19). The O₂-substituted horseradish peroxidase (1 mg/ml) was incubated with 4 × 10⁷ cells of 5 × 10⁷ cells/ml) and washed at least twice with Ca²⁺-free HBSS. The cell preparation thus obtained contained 88-97% neutrophils, and the yield was 2-10 × 10⁷ cells/ml. The cell suspension (5 × 10⁷ cells in 0.3 ml of Ca²⁺-free HBSS) in a glass culture tube (12 × 75 mm, Corning Glass Works) was frozen in liquid N₂, and stored at -70 °C. After the storage for 2 months, loss of activity was less than 10% as determined in a liquid scintillation counter (Beckman Model LS-3800) using a Triton X-100-toluene-based scintillation fluid (21).

Gas Chromatographic Analysis of Metabolites from Fatty Acids—

Metabolism of Lactate and H⁺, if any, during the activation process of the cell-free system was evaluated by gas chromatography with a Shimadzu Chromatograph equipped with a capillary column (Supelco Inc., SP-2330, 300 cm). The cell-free preparation (4 × 10⁷ cells/ml) was incubated with 2.0 mM 14C-labeled laurate (0.04 mCi/mmol, Du Pont-New England Nuclear) or 15 mM [1-¹⁴C]oleate (0.04 mCi/mmol, Amersham Corp.) for 5 min at 25 °C. The mixture was centrifuged at 200,000 × g for 5 min at 4 °C, and the surface of the resulting pellet was rinsed with Ca²⁺-free HBSS. Radioactivities in both pellet and supernatant fractions were determined in a liquid scintillation counter (Beckman Model LS-3800) using a Triton X-100-toluene-based scintillation fluid (21).

RESULTS

Stimulation of O₂-generating System in a Cell-free Preparation by Fatty Acids—Fig. 1 shows effects of various concentrations of fatty acids with the chain length from C₁₀ to C₁₄ on NADPH-dependent O₂ generation in a cell-free preparation from porcine neutrophils. As seen, saturated fatty acids exerted their maximal effects at concentrations over 2 mM, while unsaturated fatty acids had the maximal effect at around 1.5 mM and thereafter became inhibitory. In these experiments, fatty acids were added slowly under vigorous stirring to the reaction mixture which contained the cell-free preparation derived from 4 × 10⁷ cells (approximately 2 mg as protein) in 0.4 ml of Ca²⁺-free HBSS. The use of such a high concentration of the cell-free preparation was to obtain an accurate stoichiometry between O₂ consumption and O₂ generation. Under comparable conditions, the concentration of arachidonate which induced the maximal O₂ generation was also around 1.5 mM. Saturated fatty acids with longer or shorter chain length such as arachidonic acid (C₂₀) and caprylate (C₁₄) were not effective at similar concentrations.
FIG. 1. *O₂*-generating activity induced by different concentrations of fatty acids in the cell-free system. The reaction mixture (0.4 ml) contained the cell-free preparation (approximately 2 mg as protein obtained from 4 x 10⁷ neutrophils), 5 mM EGTA, 0.16-0.25 mM diacetyldeuteroheme-substituted horseradish peroxidase in Ca²⁺-free HBS, pH 7.3, at 25°C. After incubation of the mixture with an indicated concentration of fatty acid for 5 min, the reaction was initiated by the addition of 0.1 mM NADPH except for linoleate and linolenate. In the latter cases, NADPH was added immediately after the addition of fatty acid. Initial rates of *O₂* generation were determined by measuring the formation of compound III of diacetyldeuteroheme-substituted horseradish peroxidase and plotted against the fatty acid concentrations. Panel A, with saturated fatty acids: caprate (△), laurate (〇), myristate (●), palmitate (■), stearate (◇), and arachidate (▲). Panel B, with unsaturated fatty acids: palmitoleate (□), oleate (△), elaidate (◇), linoleate (▲), linolenate (▼).

By employing laurate and oleate as the representatives of saturated and monounsaturated fatty acids, we compared characteristics of the *O₂* generating reaction. The *K₅₅₅* values for NADPH in the laurate (2.0 mM)- and oleate (1.5 mM)-induced *O₂* generation were 73.4 ± 4.9 and 66.9 ± 5.6 μM, respectively, and the *V₅₅₅* values for the *O₂* generation were 0.32 ± 0.02 and 0.26 ± 0.01 mM/min, respectively (mean ± S.E. in three experiments). An approximate *K₅₅₅* for NADPH in the arachidonate (1.5 mM)-induced *O₂* generation was 50 μM, although a large amount of *O₂* consumption unrelated to *O₂* production (see below) obscured the determination of the accurate value. These *V₅₅₅* values were comparable to that induced by an optimal concentration of phorbol 12-myristate 13-acetate in intact porcine neutrophils, which was 20–30 nmol/min/10⁹ cells at 25°C. There was a low level of NADPH-consuming activity (6.8 ± 0.4 μM/min in five experiments) in the cell-free preparation in the absence of fatty acid. No significant *O₂* generation was observed by the addition of 0.1 mM NADH in both laurate- and oleate-stimulated preparation. The pH optimum was between 6.8 and 7.3 for both laurate- and oleate-stimulated activities.

**Stoichiometry between *O₂* Consumption and *O₂* Production Induced by Various Fatty Acids**—Fig. 2 shows a simultaneous record of oxygen consumption and *O₂* generation by the cell-free preparation which was activated with 2.5 mM laurate. During the preincubation with laurate for about 5 min, the preparation showed neither *O₂* consumption nor *O₂* forma-

FIG. 2. Rates of *O₂* generation and *O₂* consumption during the stimulation of the cell-free system by laurate. The reaction mixture and other conditions were the same as described under Fig. 1. The concentration of diacetyldeuteroheme-substituted horseradish peroxidase was 0.20 mM. Laurate (2.5 mM) was added at arrow a, and, after 5 min of incubation, NADPH (0.1 mM) was added at arrow b. *O₂* generation was assayed by using diacetyldeuteroheme-substituted horseradish peroxidase, and *O₂* consumption was determined polarographically (see "Materials and Methods").

Table I summarizes the initial rates of *O₂* consumption and *O₂* generation upon stimulation by various fatty acids. When the cell-free preparation was stimulated by saturated, and cis- and trans-monounsaturated fatty acids, the ratios of the two reaction rates were all approximately 1:1. The ratios of total *O₂* consumption to total *O₂* production were also about 1:1. On the other hand, the ratios of *O₂* production to *O₂* consumption were as low as 0.27, 0.53, and 0.49, when the preparation was stimulated with cis-polyunsaturated fatty acids such as arachidonate, linolenate, and linoleate, respectively. Furthermore, a massive *O₂* consumption was observed with these cis-polyunsaturated fatty acids, when they were added to the cell-free preparation in the absence of NADPH (data not shown). During such NADPH-independent *O₂* consumption, no formation of *O₂* was detected, and the amount of *O₂* consumed was roughly equal to that of the non-*O₂*-producing *O₂* consumption observed in the presence of NADPH. Thus both saturated and unsaturated fatty acids are capable of activating the *O₂*-generating system, but the latter fatty acids also induced a large amount of non-*O₂*-producing *O₂* consumption. The NADPH-independent *O₂* consumption induced by 0.25 mM linoleate was inhibited by nordihydroguaiaretic acid and BW755C lipooxygenase inhibitors, with apparent *K₅₅₅* values of 8 and 9 μM, respectively. Porcine neutrophils have been known to contain a significant amount of 12-lipoxygenase which metabolizes cis-polyunsat-
urated fatty acids such as linoleate and arachidonate (24). It is unknown, however, whether the lipoxygenase activity can account for all the O$_2$ uptake observed in the absence of NADPH, because both nordihydoxygammaeriacetic acid and BW755C are not strictly specific to the lipoxygenase reaction.

Effects of Ca$^{2+}$ on the Oxygen Metabolism in the Cell-free System—It should be noted that all of the foregoing experiments were carried out under Ca$^{2+}$-free conditions. The reason for such experimental conditions was due to the previous findings that Ca$^{2+}$ interfered with the activation of the O$_2$-generating system by fatty acids both in intact cells and in the cell-free systems (5, 6, 15, 16). Then we examined here the effects of Ca$^{2+}$ on the oxygenation by various fatty acids of O$_2$-generating system in the present cell-free system. As seen in Fig. 3, the laurate-induced O$_2$ generation was completely inhibited by the presence of 1.0 mM Ca$^{2+}$, while O$_2$ generation with linoleate or arachidonate was scarcely affected by the same concentration of Ca$^{2+}$. Intermediate degree of inhibition was observed with oleate- and linoleate-induced systems. In these experiments, O$_2$ generation was initiated by the addition of NADPH immediately after the addition of a fatty acid. When NADPH was added at 5 min after the addition of a fatty acid in the presence of Ca$^{2+}$, however, even linoleate- or arachidonate-induced O$_2$ generation was inhibited (see below).

In the next experiments, we examined the effects of Ca$^{2+}$ on the O$_2$-generating system which had been activated by the incubation with a fatty acid for 5 min. At 1 min after the addition of Ca$^{2+}$, none of the O$_2$-generating activity induced by both saturated and unsaturated fatty acid was affected. After 5 min of incubation with Ca$^{2+}$, however, the activity was found to be decreased significantly, the laurate- and linoleate-induced O$_2$ generation decreased to 30 and 60% of their original activities, respectively. It should be noted that the activity induced by saturated fatty acids was more affected than that by unsaturated fatty acids. The reason(s) for this phenomenon will be discussed in detail under "Discussion."

Ca$^{2+}$ had another effect on the oxygen metabolism in the present system. When the cell-free preparation was incubated with cis- or trans-unsaturated fatty acid in the presence of Ca$^{2+}$, a significant amount of O$_2$ consumption was induced in the absence of NADPH. For example, the rate of O$_2$ consumption in the absence of exogenous NADPH was 82 ± 13 µM/min (mean ± S.E. in 10 experiments), when the system was stimulated with 0.5-1.0 mM oleate in the presence of 2 mM Ca$^{2+}$. The concentration of Ca$^{2+}$ required for a half-maximal activation was 0.03 mM. The O$_2$ consumption was not affected by up to 2 mM of Na$_2$S$_2$O$_4$ and KCN, and formation of O$_2$ or H$_2$O$_2$ was not detected during the process. No similar O$_2$ consumption in the absence of NADPH was observed with saturated fatty acids nor with the sonicates of porcine lymphocytes and platelets. We also found that at a pH lower than 6.5, all kinds of fatty acids including saturated fatty acids elicited an NADPH-independent O$_2$ consumption even in the absence of Ca$^{2+}$.

| Fatty Acid (chain length) and concentration employed | Rate of reactions | Ratio O$_2$/O$_2$ |
|---------------------------------------------------|------------------|-----------------|
|                                                   | μM/min           | μM/min × 10$^{-2}$ |                  |
| Saturated fatty acid                              |                  |                 |
| Caprylate (C$_4$H$_8$)                             | 30               | <0.01           |
| Caprate (C$_{10}$H$_{20}$)                         | 12               | 0.33            |
| Laurate (C$_{12}$H$_{24}$)                         | 2.5              | 2.28 ± 0.09     |
| Myristate (C$_{14}$H$_{28}$)                        | 2.5              | 3.34 ± 0.26     |
| Palmitate (C$_{16}$H$_{34}$)                        | 2.0              | 1.31 ± 0.12     |
| Stearate (C$_{18}$H$_{36}$)                         | 1.5              | 0.09            |
| Arachidate (C$_{20}$H$_{40}$)                       | 1.5              | <0.01           |
| Unsaturated fatty acid                             |                  |                 |
| Elaidate (t-C$_{18}$H$_{36}$)                       | 1.5              | 1.39 ± 0.21     |
| Palmitoleate (e-C$_{18}$H$_{36}$)                   | 1.5              | 1.90 ± 0.19     |
| Oleate (C$_{18}$H$_{36}$)                           | 1.5              | 1.91 ± 0.12     |
| Linoleate (C$_{18}$H$_{36}$)                        | 1.5              | 1.88 ± 0.16     |
| Linolenate (C$_{18}$H$_{36}$)                       | 1.5              | 2.09 ± 0.15     |
| Arachidonate (C$_{20}$H$_{42}$)                     | 1.5              | 2.80 ± 0.70     |

$^*$ Fatty acid concentrations which gave maximal activation were employed.

$^*$ Mean ± S.E. obtained from at least three independent experiments.

$^*$ t- and e- denote trans- and cis-configuration, respectively.

![Fig. 3. Effect of Ca$^{2+}$ on the O$_2$-generating activity induced by various fatty acids in the cell-free system.](image-url)
Requirement of Soluble Factor(s) for the Activation—As described earlier, saturated and monounsaturated fatty acids activated specifically the $O_2^-$ production in the cell-free preparation from the neutrophils. We therefore tried to characterize further the system by fractionating it into the membrane and soluble fractions (Table II). When the cell-free preparation was first activated by laurate (C12:0) or oleate (C18:1) and then separated into membrane and soluble fractions by centrifugation, the membrane fraction was found to be fully active in producing $O_2^-$ upon addition of NADPH. In contrast, the soluble fraction had neither $O_2^-$ producing activity nor any effect on the activity of the activated membrane fraction (Table II, Experiment A). On the other hand, when the system was first separated into membrane and soluble fractions and each of them was incubated separately with laurate or oleate, $O_2^-$-generating activity was found in neither fractions. When the two fractions were combined and incubated with the fatty acid, a full activation of the $O_2^-$-generating system was obtained (Experiment B). Similar results were obtained upon activation of the system with other fatty acids such as myristate and a trans-monounsaturated fatty acid, elaidate. These results are in agreement with those of others who demonstrated the requirement of a soluble fraction in the activation of $O_2^-$-generating system in cell-free preparations from neutrophils or macrophages by unsaturated fatty acids and sodium dodecyl sulfate (5-11).

We found, however, that a soluble fraction obtained from the cell-free preparations previously activated by fatty acids was no longer effective even when it was combined with a fresh membrane fraction and incubated with the same or different fatty acids used for the prior activation (Table II, Experiment C). Thus the effective component for the activation was lost from the soluble fraction during the activation.

The loss of activity was also observed by using other fatty acids. Additions of ATP (0.01-1.0 mM) or GTP (0.01-1.0 mM) to the used soluble fraction had no effect on the lost activity. A similar loss of the active component(s) seems to take place in vivo, since a soluble fraction obtained from intact neutrophils which were activated with laurate (0.45 mM) or phorbol 12-myristate 13-acetate (200 ng/ml) for 5 min was less effective than that from untreated cells by 40-60% in stimulating the membrane-bound $O_2^-$-generating system. The viability of these activated neutrophils was more than 95% as judged by trypan blue dye exclusion test.

Reversible Activation of $O_2^-$-generating System—When the cell-free preparation was activated by a fatty acid and then incubated with a sufficient amount of delipidated albumin, the activity decreased with increasing time of incubation resulting in an almost complete deactivation within 10 min (Fig. 4, insets). As seen, both laurate (2.0 mM)- and oleate (1.5 mM)-induced activities were completely lost by the incubation with 0.2-0.3 mM albumin. Similar deactivation by delipidated albumin was observed with the membrane fraction isolated from the cell-free preparation which had been activated by 1.5 mM oleate or 2.0 mM laurate. Fatty acid-saturated albumin (0.5 mM) had no inhibitory effect on either the oleate-or laurate-induced $O_2^-$-generating activity. These results, together with those of Bedwey et al. (2) on intact neutrophils, suggest that the inhibitory effect of delipidated albumin was caused by the removal of fatty acid molecules from the activated membranes, and this was confirmed by the following experiments using radiolabeled fatty acids. When the system was preincubated with [1-14C]laurate or [1-14C]oleate and then treated with the delipidated albumin for 10 min, most of the

TABLE II

Requirement of fresh soluble fraction for the activation of $O_2^-$-generating system and localization of the activated system

In Experiment A, the membrane and soluble fractions were obtained from a cell-free preparation which had been activated with 2.5 mM laurate or 1.5 mM oleate, and were assayed for $O_2^-$ generation either separately or together. In Experiment B, the membrane, soluble or their combined fraction from an unstimulated cell-free preparation, was incubated with 2.5 mM laurate or 1.5 mM oleate for 5 min and was assayed for $O_2^-$ generation. In Experiment C, a membrane fraction from unstimulated cell-free preparation was combined with fresh or used soluble fractions, and their activities after incubation with 2.5 mM laurate for 1.5 mM oleate for 5 min were determined. The amounts of cell-free preparations, soluble and membrane fractions, used were all that amount derived from 4 x 10^9 cells. The reaction mixture (0.4 ml) was as same as that in Fig. 2. Reaction and assay conditions were also as in Fig. 2. Control activities were those of nonfractionated cell-free preparations activated by the same amount of respective fatty acids.

| Experiment | Fraction | Relative activity of $O_2^-$ generation* |
|------------|----------|-----------------------------------------|
| A. Localization of the activated system | Membrane | Soluble | Laurate | Oleate |
| + | - | 0.97 ± 0.05 | 0.93 ± 0.03 |
| - | + | 0.94 ± 0.02 | 0.93 ± 0.03 |
| B. Effect of fresh soluble fraction in the activation | + | - | 0.02 ± 0.006 | 0.05 ± 0.004 |
| - | + | 0 | 0 |
| + | + | 0.95 ± 0.08 | 0.83 ± 0.10 |
| C. Ineffectiveness of used soluble fraction | + | * | 0.91 ± 0.07 | 0.87 ± 0.08 |
| + | *+ | 0.11 ± 0.02 | 0.14 ± 0.03 |
| + | *+ | 0.11 ± 0.03 | 0.15 ± 0.03 |

*Activities (mean ± S.E. from three independent experiments) were expressed as ratios to control activities which were taken as 1.0. The control activities were 175 ± 10 and 155 ± 9 for Experiment A, 157 ± 8 and 174 ± 12 for Experiment B, and 197 ± 7 and 174 ± 12 µM/min for Experiment C, by laurate and oleate, respectively.

Fresh soluble fraction obtained from a nontreated cell-free preparation as in Experiment B.

Obtained from a cell-free preparation activated by laurate as in Experiment A.

Obtained from a cell-free preparation activated by oleate as in Experiment A.
The cell-free preparation was incubated with 14C-labeled laurate (2.0 mM) or oleate (1.5 mM) for 5 min at 25 °C and then incubated with 0.3 mM delipidated albumin for 10 min at 25 °C. Distribution of radiolabeled fatty acids was determined before and after the albumin treatment, and the values were expressed as percent recovery. The membrane and soluble fractions were isolated by centrifugation at 200,000 × g for 30 min at 4 °C (Experiment A) or at 25 °C (Experiment B). Each value is the mean from duplicate experiments.

| Albumin treatment | Distribution | % recovery |
|-------------------|--------------|------------|
|                    | Membrane    | Supernatant| Membrane | Supernatant|
| Experiment A       | Before       | 69.4       | 29.7      | 92.3       | 3.5        |
|                    | After        | 3.6        | 96.4      | 4.1        | 96.1       |
| Experiment B       | Before       | 70.4       | 29.4      | 94.5       | 2.7        |
|                    | After        | 1.8        | 99.6      | 3.9        | 94.3       |

**DISCUSSION**

We have shown in this study that the saturated and monounsaturated fatty acids are both good stimuli for the activation of the O2-generating system in a cell-free preparation from porcine neutrophils. As judged by the stoichiometric relationship between O2 consumption and O2 formation in the reaction, these fatty acids activate specifically the O2-generating system in the cell-free preparation. The results, together with the previous findings by Kakinuma and coworkers (12–15) with intact phagocytes, have unambiguously established that the activation of the phagocyte O2-generating system by fatty acids is not limited to that by cis-unsaturated fatty acids.

The reason why most of previous investigators have overlooked the effect of saturated fatty acids on the O2-generating system both in vivo and in vitro is not clear, but the following points may be discussed as possibilities. 1) Ca2+, which was often included in the reaction mixtures of the past experiments, interacts more easily with saturated fatty acids than with unsaturated fatty acids to alter their solubilities (17), which in turn affect the effective concentration of fatty acids in the reaction mixture. It has been pointed out that Ca2+ elevates the Kraft point of a fatty acid (15). Alternatively, Ca2+ can interact with saturated fatty acids in the membrane, for example, to form copolymeric lattice structure (18), which may harden the membrane to reduce its permeability. 2) The method of adding fatty acids is critical. As we have mentioned in this paper, a fatty acid, especially a saturated fatty acid, must be added to the reaction mixture slowly at a constant rate under vigorous stirring. If not, much less activation or irreproducible results were obtained, presumably due to the formation of fatty acid micelles or aggregates, which may reduce effective interactions of the fatty acid with the enzyme system in the membrane and/or with the soluble component(s).

The mechanism(s) by which fatty acids activate the O2-generating system has been the subject of considerable investigation. For example, Badwey et al. (2) have suggested physicochemical changes in the plasma membrane induced by the intercalation of a cis-unsaturated fatty acid, while Bromberg and Pick (11) have raised various possibilities primarily attributable to the detergent action of sodium dodecyl sulfate. The role of guanine nucleotide-binding protein has also been emphasized by many workers (9, 10, 25, 26), who suggested that fatty acids facilitate interactions among membrane-bound oxidase, soluble component(s), and the guanine nucleotide-binding protein(s). Then the present study provides the following insights into understanding the mechanism(s) of the activation by fatty acids of neutrophil O2-generating system.

First, the finding that the laurate and oleate, most frequently used as stimuli in this study, were not metabolized in our cell-free system indicates that the fatty acids themselves and not their metabolites are responsible for the activation. Second, the fatty acid must remain in the membrane to maintain the activated state of the O2-generating system both in vivo and in vitro. Karnovsky and his associates (2) have shown that intact neutrophils activated by arachidonate can be deactivated by the addition of delipidated albumin and reactivated by the replenishment of the fatty acid. We demonstrated here that the removal and the replenishment of the fatty acid from and to the membrane preparation deactivated and reactivated recurrently the O2-generating system in a cell-free state. Third, a soluble fraction was required also for the activation by saturated fatty acids. Similar requirement of a soluble fraction has been pointed out for the unsaturated

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**TABLE III**

**Removal of radiolabeled fatty acids from the membrane fraction by albumin treatment**

| Albumin treatment | Distribution | % recovery |
|-------------------|--------------|------------|
|                    | Membrane    | Supernatant| Membrane | Supernatant|
| Experiment A       | Before       | 69.4       | 29.7      | 92.3       | 3.5        |
|                    | After        | 3.6        | 96.4      | 4.1        | 96.1       |
| Experiment B       | Before       | 70.4       | 29.4      | 94.5       | 2.7        |
|                    | After        | 1.8        | 99.6      | 3.9        | 94.3       |

**TABLE IV**

**Effects of fresh soluble fraction on the restoration of O2-generating activity in albumin-treated membrane fraction**

The cell-free preparation obtained from 4 × 107 cells which had been activated by laurate, myristate, oleate, or elaidate was deactivated by the incubation with delipidated albumin. The albumin concentrations used were 0.38 mM for laurate- and myristate-activated preparations and 0.3 mM for oleate- and elaidate-activated preparations. Then the albumin-treated membrane fraction was obtained by centrifugation at 400,000 × g and reactivated by fatty acids in the presence or absence of a fresh soluble fraction (derived from 4 × 107 cells). Fatty acid concentrations used for both preactivation and reactivation were 2.5 mM for laurate and myristate or 1.5 mM for oleate and elaidate. Other conditions and assay methods were the same as those in Fig. 1.

| Fraction | Rate of O2 generation restored by the addition of fatty acid | % activity |
|----------|------------------------------------------------------------|------------|
|          | Membrane Soluble | Laurate Myristate Oleate Elaidate |          |
| +        | -                | 3.7 ± 0.9* | 2.6* | 13.5 ± 4.2 | 8.7       |
| +        | +                | 41.2 ± 4.1 | 77.2 | 53.1 ± 2.7 | 61.8      |

* Percent of control O2-generating activities, which were 175 ± 10 (n = 3), 298, 155 ± 9 (n = 3), and 173 μM/min obtained by laurate-, myristate-, oleate-, and elaidate-activated cell-free preparations before albumin treatment, respectively.

**From single experiments.
fatty acid- and sodium dodecyl sulfate-mediated activation of the O₂-generating system (5–11, 25–28). Fourth, the effect of the soluble fraction was lost during the incubation, suggesting that the effective component(s) was translocated to the membrane or consumed during the incubation. At the moment, it is not known whether the activation mechanism in our cell-free system is different from or identical to that in intact cells. However, at least a soluble component appears to be involved in the activation process commonly in vitro and in vivo. Loss of activity in the soluble component(s), when it was once used for the activation, was observed not only in the cell-free system but also in intact neutrophils. Reversibility of the fatty acid-induced O₂ production, which was observed both in vitro (present paper) and in vivo (2), may also favor the view that the system is activated via a common mechanism. These findings, together with a recent observation by Curnutte and co-workers (29, 30) that a soluble factor(s) was deficient in neutrophils of an autosomal CGD patient whose neutrophils contained a normal level of cytochrome b₅₅₆ but had severely damaged O₂-generating ability, may indicate the pivotal importance of such a soluble factor in the activation of the O₂-generating system. Then what is the mechanism(s) for the cooperation of the soluble component(s) with a fatty acid? If the soluble component(s) was really translocated to the membrane, the fatty acids may facilitate the binding of the component to the membrane, mimicking the anchorage process of a soluble protein such as by fatty acylation (31) or phosphatidylinositoloylation (32). Translocation of proteins such as protein kinase C (Ca²⁺- and phospholipid-dependent protein kinase) or diacylglycerol kinase to the membrane and their dissociation therefrom are considered to be important mechanisms for controlling the activity of membrane enzyme system (33, 34).

Finally, a massive O₂ consumption induced by poly- and monounsaturated fatty acids is of interest. The O₂ consumption yielded neither O₂⁻ nor H₂O₂, and can be subgrouped into Ca²⁺-dependent and -independent processes. As mentioned, they might not be explained solely by the lipoxigenase reaction. Identification of such O₂ consuming reactions as well as their significance in the O₂ metabolism in neutrophils remains to be elucidated.

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