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DIACYLGLYCEROL, 1-OLEOYL-2-ACETYL-GLYCEROL, STIMULATES SUPEROXIDE-GENERATION FROM HUMAN NEUTROPHILS

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SUMMARY: 1-Oleoyl-2-acetyl-glycerol which activates Ca^{2+}-activated phospholipid-dependent protein kinase, induced the superoxide-production of human neutrophils, while other diacylglycerols did not. The induction was independent of extracellular calcium and did not accompany the increase of the intracellular free calcium. The superoxide-release by the diacylglycerol was inhibited by retinal, the inhibitor of the protein kinase. The diacylglycerol stimulated the phosphorylation of at least 4 proteins in intact neutrophils, the phosphorylation of which was stimulated by phorbol 12-myristate 13-acetate, the activator of the protein kinase. These observations indicate the possible involvement of the kinase in the induction process.

Neutrophils generate superoxide anion (O_2^-) during phagocytosis or by the stimulation of the cells with reagents such as phorbol 12-myristate 13-acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (FMLP). The superoxide-generation is considered to be catalyzed by an NADPH oxidase (1) which is dormant in the resting cells and activated by an increase in intracellular free calcium(2,3). Involvement of calmodulin in the activation was suggested, based on the finding that calmodulin inhibitors such as trifluoperazine and N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) inhibited the activation (4), but the mechanism remains unknown. Recently, a regulatory system dependent on calcium, Ca^{2+}-activated, phospholipid-dependent protein kinase (C-kinase) which can also be inhibited by trifluoperazine and W-7, was shown in neutrophils and many other cells (5-7). Although C-kinase is directly activated by diacylglycerols (8), products of phosphatidyl inositol

Abbreviations used: OAG, 1-oleoyl-2-acetyl-glycerol; C-kinase, Ca^{2+}-activated phospholipid-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; EGTA, bis-(3-aminoethylether)-N,N,N',N'-tetraacetic acid; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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turnover (9), only a synthesized diacylglycerol, 1-oleoyl-2-acetyl-glycerol (OAG) can activate C-kinase in intact platelets (10). Because C-kinase is activated by PMA with the increase of the affinity to calcium(11) and neutrophils were activated by PMA without an increase in intracellular free calcium, the involvement of C-kinase in the activation was suggested (12).

In this paper, we report the induction of superoxide-formation by OAG in human neutrophils and its inhibition by retinal, known to inhibit C-kinase. The phosphorylation of proteins in the intact cells was also stimulated by OAG. These results support the idea that C-kinase is involved in the induction of the superoxide-forming system.

EXPERIMENTAL

Human neutrophils were prepared from peripheral blood as described previously (1) except that polyvinylpyrrolidone was replaced by dextran. The activity of the cells to release superoxide was measured by the reduction of cytochrome c. The assay mixture (1.0 ml) consisted of 7.5 μM ferricytochrome c and 1.5×10^6 cells in a buffer (137 mM-NaCl, 4.9 mM-KCl, 0.5 mM-$CaCl_2$, 1.2 mM-$MgSO_4$, 5.5 mM-glucose and 5.7 mM-sodium phosphate buffer, PH 7.4). After the cells were incubated at 37°C for 10 min, the reaction was started by the addition of a stimulant and monitored by a Hitachi dual-wavelength spectrophotometer at 550-540 nm. The rate of the superoxide-release was calculated from the linear portion of cytochrome c reduction using a molar absorption coefficient of 19 100 $M^{-1}cm^{-1}$. Intracellular free calcium was measured using a calcium-sensitive fluorescent probe, quin 2, by the method of Sha'afi et al.(12). Two-dimensional gel-electrophoresis was performed by the method of O'Farrell with slight modifications (13). Proteins of ^{32}P-loaded neutrophils stimulated by OAG or PMA were electrophoresed on isoelectric-focuing gels for the first dimension and then 13.5% polyacrylamide gels containing sodium dodecyl sulphate for the second dimension, stained with Coomassie Brilliant Blue and autoradiographed.

OAG and 1-oleoyl-2-stearoyl-glycerol synthesized by the method of Buchnea (14) were generous gifts from Eisai Pharmaceutical Co.(Tokyo, Japan). 1,2-Diolein, retinal, PMA, cytochrome c and superoxide dismutase were purchased from Sigma Chem.Co.(St.Louis.Mo, USA). 1,2-Dilinolein, oleate and FMLP were from Funakoshi Chem.Co.(Tokyo, Japan), Nakarai Chem.Co.(Kyoto, Japan) and the Peptide Institute (Osaka, Japan), respectively. Dibutylyl cAMP (Bu_2-cAMP) and dibutylyl cGMP (Bu_2-cGMP) were from Boehringer Mannheim (W.Germany). Ethyleneglycol bis-(3-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) and quin 2/AM were obtained from Dojin-Kagaku Laboratory (Kumamoto, Japan). $H_3{}^{32}PO_4$ was obtained from Nihon-Genshiryoku Institute (Tokyo, Japan). Other reagents were of analytical grade. OAG, 1-oleoyl-2-stearoyl-glycerol, diolein, dilinolein, retinal, PMA and FMLP were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the assay mixture was 1%. Experiments with retinal were carried out under subdued light.

RESULTS AND DISCUSSION

On exposure to OAG, human neutrophils released superoxide as detected by the reduction of cytochrome c, which was completely inhibited by superoxide

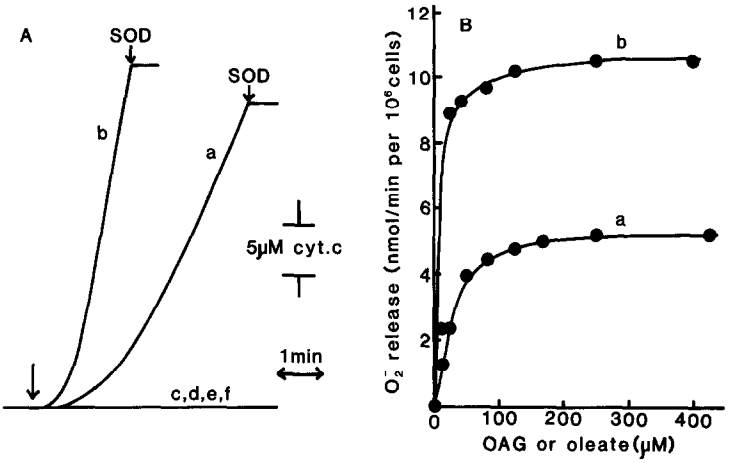

Fig. 1. Superoxide-release of human neutrophils stimulated by diacylglycerols and oleate.
A) Time course of the O_2^--release stimulated by diacylglycerols and oleate. The activities were completely inhibited by superoxide dismutase (SOD). The reaction was started by the addition of a stimulant (indicated by an arrow). a) OAG (250 μM), b) oleate (250 μM), c) diolein (0.1 mg/ml), d) dilinolein (0.1 mg/ml), e) 1-oleoyl-2-stearoyl-glycerol (0.1 mg/ml). f) no addition.
B) Dose-dependent curves of the O_2^--release stimulated by a) OAG and b) oleate.

dismutase (Fig. 1A, a). The maximal rate was 5.2 nmol O_2^-/min per 10^6 cells with a lag time of about 3 minutes. In contrast, other diacylglycerols, such as diolein, dilinolein and 1-oleoyl-2-stearoyl-glycerol, did not induce the release, in agreement with the report on platelets (10) which can be activated by OAG but not by other diacylglycerols with two long fatty acyl moieties known to activate C-kinase. Permeabilities or some other properties of the diacylglycerols may be responsible for the difference.

Unsaturated fatty acids have been shown to induce the superoxide-formation in neutrophils (15,16). Oleate activated the formation with shorter lag time of about 30 sec (Fig. 1A,b) and the concentration of oleate for half-maximal rate was 18 μM and was lower than that of OAG (30 μM)(Fig. 1B). A possibility should be considered that the oleate released from OAG, not OAG itself, stimulates the cells, but it is unlikely because the maximal rate of the superoxide formation induced by saturated concentration of OAG was about half of the maximal rate with oleate. This was further supported by experiments given in Table I, in which the effects of 4 inhibitors, EGTA, cyclic

Table 1. Effects of Ca^{2+} and cyclic nucleotides on O_2^- release of neutrophils

Stimulants	Control	$-Ca^{2+}$	+EGTA	$+Bu_2$-cAMP	$+Bu_2$-cGMP	+DOG
O A G (250 μM)	4.9±0.4	4.7±0.5	4.5±0.3	4.5±0.4	4.3±0.3	0.7±0.1
P M A (1 μg/ml)	11.3±0.4	10.4±2.1	11.1±1.7	10.1±1.0	10.5±2.2	2.1±0.1
Oleate (250 μM)	11.0±1.3	6.9±0.9	7.2±1.1	12.3±0.9	9.3±0.5	1.1±0.1
F M L P (1 μM)	4.1±0.8	1.7±1.0	1.2±0.7	1.0±0.4	2.0±0.6	0.1±0.1

After the cells were preincubated with EGTA (4 mM), Bu_2-cAMP(1 mM) and theophylline (0.5 mM), Bu_2-cGMP (1 mM) or 2-deoxyglucose (DOG:5.5 mM) in the assay mixture containing 0.5 mM Ca^{2+} at 37°C for 10 min, the reaction was started by the addition of a stimulant. Values represent the superoxide release (nmol/min per 10^6 cells, mean ± S.D.) of four independent experiments.

nucleotides and deoxyglucose, on the superoxide-formation induced by 4 stimulants, OAG, PMA, oleate and FMLP, were compared. The superoxide formation stimulated by OAG or PMA was essentially not inhibited by EGTA, Bu_2-cAMP and Bu_2-cGMP, while the formation induced by FMLP, which has been shown to evoke the phosphatidyl inositol turnover, was clearly inhibited by EGTA and cyclic nucleotides. The formation induced by oleate was also inhibited by EGTA, but not by the cyclic nucleotides. The results suggest that the mechanism of the stimulation by OAG is very similar to that by PMA, but not to those by FMLP and oleate.

The similarity of the induction by OAG to that by PMA was observed in the changes of intracellular free calcium concentrations measured by using a fluorescent probe quin-2 (Fig. 2). No increase in fluorescence was observed when

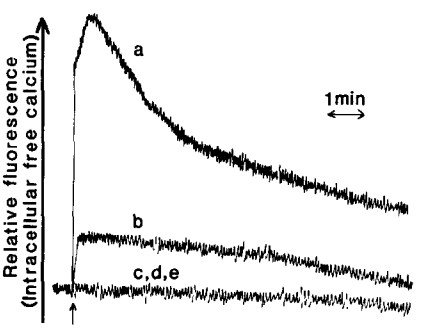

Fig. 2. Effects of OAG, PMA, FMLP and oleate on intracellular free Ca^{2+}. Intracellular free Ca^{2+} was measured by using quin 2 fluorescence in modified Hanks' Balanced Salt Solution containing 1 mM-Ca^{2+} and 1 mM-Mg^{2+}. After the preincubation for 10 min at 37°C, the reaction was started by the addition of a stimulant (indicated by an arrow). a) FMLP (1 μM), b) PMA (1 μg/ml), c) OAG (250 μM), d) PMA (0.1 μg/ml), e) no addition.

the cells were stimulated by OAG (curve c) or PMA at a saturated concentration for the superoxide production (0.1 μg/ml: curve d), while FMLP caused a substantial increase in fluorescence (curve a) and PMA at 1 μg/ml caused a slight increase (curve b), in agreement with the report of Sha'afi et al. (12). The results suggest that the increase of intracellular free calcium is not essential by itself in the induction process but the C-kinase, which can be activated by diacylglycerols in the absence of the increase in intracellular free calcium, may be involved in the induction.

We have previously shown that the induction of superoxide formation by various stimulants was inhibited by trifluoperazine and W-7 (4), which are now known as inhibitors of C-kinase. Another inhibitor of C-kinase, retinal, also inhibited the induction by various stimulants. The concentration of required for 50% inhibition of the superoxide formation stimulated by FMLP, PMA, OAG and oleate were 11 μM, 9 μM, 7 μM and 5 μM, respectively (Fig. 3), in agreement with an in vitro Ki of PMA-activated C-kinase, 10 μM (17). The inhibitor did not inhibit the reduction of cytochrome c by xanthine-xanthine oxidase system, inidicating that it is not a scavenger of superoxide radicals.

We also studied the phosphorylation of proteins in intact neutrophils preloaded with radioactive phosphate and stimulated by OAG. The radioactivities of several proteins in the stimulated cells were enhanced compared with

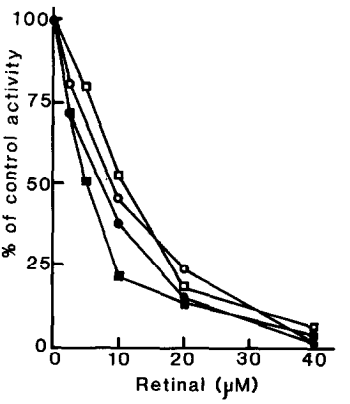

Fig. 3. Effects of retinal on the superoxide release.
Cells were preincubated with inhibitor for 10 min at 37°C, and then stimulated. The inhibition is given as % of control activity. Stimulants used were OAG (●), PMA (○), FMLP (□) and oleate (■).

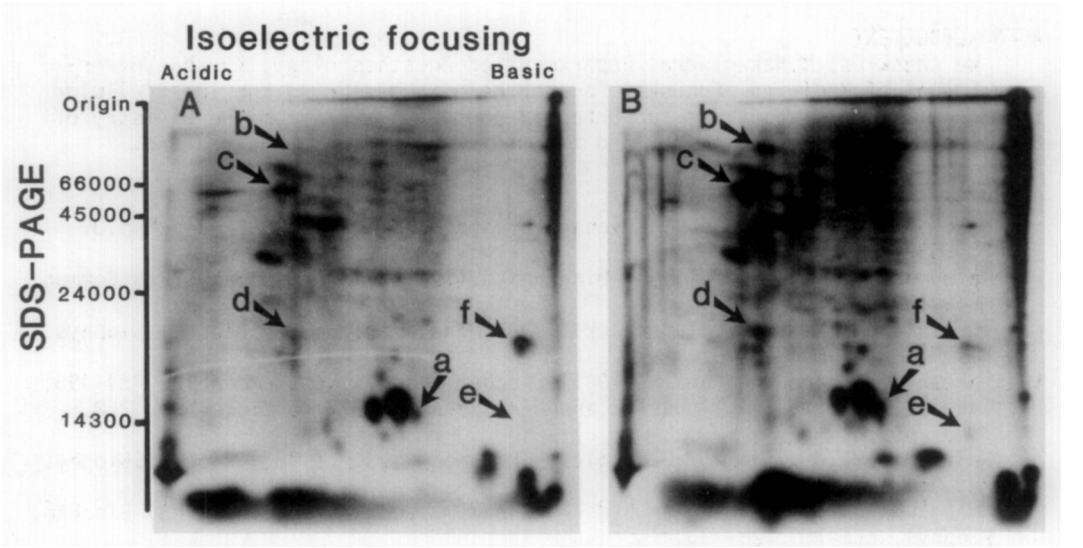

Fig. 4. Two-dimensional gel electrophoresis of phosphoproteins in neutrophils. Proteins of ^{32}P-loaded neutrophils were electrophoresed on isoelectric focusing gels for the first dimension and 13.5% polyacrylamide gels containing sodium dodecyl sulphate (SDS-PAGE) for the second dimension. The gels were then autoradiographed at -70°C for 7 days. A) resting cells, B) OAG-treated cells.

those in the resting cells (Fig. 4). One of them (spots a, Mr: 15 000) may correspond to the one reported by Helfman et al. (6) as the *in vitro* substrates of C-kinase in the particulate fraction of human neutrophils (Mr: 89 000, 38 000, 34 000, 17 000 and 15 000). Other spots, the radioactivities of which were enhanced in the cells stimulated by OAG, were Mr:98 000, 62 000, 20 000 and 13 000 (spots b, c, d, e). The radioactivities of these spots were also enhanced by stimulating the cells with PMA. The phosphorylation of these proteins was inhibited in the neutrophils treated with retinal before the incubation with OAG (not shown). Decreased radioactivity of a spot with Mr: 19 000 was observed in the stimulated cells (spot f), but we do not know whether it is due to the dephosphorylation or the degradation of the protein. Superoxide produced by xanthine-xanthine oxidase system did not stimulate the phosphorylation of proteins in resting cells and the phosphorylation in the presence of OAG was not inhibited by superoxide dismutase(10 μg/ml).

The observations reported here may serve as evidence supporting the possible involvement of C-kinase in the activation of the superoxide-forming system in neutrophils.

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