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Activation Mechanism of Arachidonic Acid in Human Neutrophil Function*

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ABSTRACT

In Ca^{++} containing media, arachidonic acid markedly stimulated superoxide and H_2O_2 generation and activated NADPH oxidase. In Ca^{++} free media, stimulatory action of arachidonic acid on NADPH oxidase was not detected. Arachidonic acid-stimulated respiratory burst was inhibited by EGTA, TMB-8, verapamil, diltiazem, nifedipine, dibucaine, lidocaine, CCCP, 2,4-dinitrophenol, sodium arsenate, chlorpromazine, theophylline, $HgCl_2$, PCMB and PCMBSA but not affected by tetrodotoxin, tetraethylammonium chloride and procaine. EGTA almost completely inhibited release of β-glucuronidase by arachidonic acid and verapamil, CCCP and theophylline slightly inhibited it, whereas dibucaine did not show any significant effect. Arachidonic acid induced Ca^{++} release from intact neutrophils and it was decreased by TMB-8. Arachidonic acid-induced elevation of intracellular free Ca^{++} level was inhibited by EGTA and CCCP and slightly inhibited by TMB-8. Amount of intracellular free Ca^{++} increased by either arachidonic acid plus verapamil or arachidonic acid plus dibucaine was greater than that by arachidonic acid alone.

These results suggest that various changes of biochemical events may be implicated in the functional expression in neutrophils activated by arachidonic acid. Arachidonic acid appears to elevate cytosolic free Ca^{++} level by stimulating Ca^{++} release from intracellular Ca^{++} storage sites. During activation of neutrophils, Ca^{++} influx and efflux may be accomplished, simultaneously.

Key Words: Arachidonic acid, Respiratory burst, Degranulation, Calcium mobilization(Human neutrophils)

INTRODUCTION

cis-Unsaturated fatty acids including arachidonic acid stimulate neutrophil's functions, the mobilization of calcium (Sha'afi *et al.*, 1980), the polymerization of actin (Yassin *et al.*, 1985), degranulation (Naccache *et al.*, 1979), aggregation (O'flaherty *et al.*, 1979) and the stimulation of superoxide generation (Badwey *et al.*, 1981). Particularly, arachidonic acid, which is liberated from phospholipid of neutrophil's plasma membrane and tissues in inflammed sites, is known to have the unique property of causing activation of the respiratory burst without the lag time characteristic of other neutrophil stimuli (Badwey *et al.*, 1984: Curnutte *et al.*, 1984). cis-Unsaturated fatty acids readily intercalate into the plasma membranes of cells and then affect protein-lipid interactions (Klausner *et al.*, 1980). This interaction results in significant pertubation of the membrane with consequent functional changes. Fatty acids may alter the physical properties of membranes (Klausner *et al.*, 1980), induce membrane fusion (Ahkong *et al.*, 1973), activate enzymes (Gietzen *et al.*, 1982: Takenawa and Nagai, 1982), bind covalently to proteins (Agrawal *et al.*, 1983) and induce alterations of the cytoskeleton and contractile proteins (Hoo-

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ver *et al.*, 1981). Arachidonic acid is capable of activating the superoxide (O_2^-)-forming NADPH oxidase in postnuclear supernatants of sonicated or homogenized resting neutrophils (Curnutte, 1985) or macrophages (Bromberg and Pick, 1984). It is suggested that arachidonic acid may activate the respiratory burst by effecting membrane fluidity changes in the locale of NADPH oxidase (Badwey *et al.*, 1984). Certain detergents has also a similar effect on NADPH oxidase from neutrophils and macrophages. Arachidonic acid and sodium dodecyl sulfate appear to activate oxidase by their detergent properties (Bromberg and Pick, 1985). However, it is also reported that arachidonic acid does not influence microsomal membrane permeability (Chan and Turk, 1987).

Several lines of evidence postulate that arachidonic acid itself may play a role as a second messenger in the regulation of cell functions (McPhail *et al.*, 1984). Protein kinase C (Murakami and Routtenberg, 1985) and phospholipase C (Takenawa and Nagai, 1982) have been shown to be activated by arachidonic acid. In addition, arachidonic acid has also been reported to induce release of Ca^{++} in permeabilized neutrophils (Beaumier *et al.*, 1987) and hepatic microsomes (Chan and Turk, 1987). In hepatic microsomes, arachidonic acid-induced Ca^{++} release is not affected by inhibitors of arachidonate metabolism. Arachidonic acid markedly elevates cytosolic free Ca^{++} level in intact neutrophils, but in this case, the mechanism of calcium mobilization is complex (Naccache *et al.*, 1989). There is also a poor correlation between intact cell activation with arachidonic acid and that observed with broken cells (Maridonneau-Parini and Tauber, 1986).

Since the biochemical mechanism by which arachidonic acid stimulates functional response of neutrophils and role of Ca^{++} in respiratory burst in uncertain, influences of Ca^{++} chelators, ionic channel blockers, local anesthetics and other compounds on functional response in arachidonic acid-activated neutrophils were investigated in this study. Calcium mobilization in response to arachidonic acid was also observed.

MATERIALS AND METHODS

Arachidonic acid, ethyleneglycol-bis (β-aminoethyleter), N,N,N'N',-tetraacetic acid (EGTA), verapamil, diltiazem, nifedipine, tetrodotoxin (TTX), tetraethylammonium chloride (TEA), dibucaine, lidocaine, procaine, p-chloromercuribenzoic acid (PCMB), p-chloromercuribenzene sulfonic acid (PCMBSA), carbonyl cyanide m-chlorophenylydrazone (CCCP), 2,4-dinitrophenol (2,4-DNP), sodium arsenate, chlorpromazine, ferricytochrome c, scopoletin, phenolphthalein-glucuronic acid and quin 2/AM were purchased from Sigma Chemical Co.. 8- (Diethylamino) octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) and theophylline were obtained from Aldrich Chemical Co.; Murexide from J.T. Backer Chemical Co.. Other chemicals were of analytical reagent grade.

Preparation of neutrophils

Neutrophils were isolated from ACD treated venous blood of healthy donors by dextran (average molecular weight 465,000) sedimentation of erythrocytes and treatment with 0.85% ammonium chloride (Trush *et al.*, 1978). The purity of neutrophil suspensions averaged 90% as judged by Wright-Giemsa stain.

Preparation of NADPH oxidase containing granule rich fraction in neutrophils

Neutrophils were suspended in 0.25 M sucrose to a concentration of 10^8 cells/ml. The cell suspension was disrupted by sonication for three 15 sec intervals at 25 watts power with a Branson sonifier cell disruptor (Mod. W185D). Unbroken cells and nuclei were sedimented by centrifugation at 800 g for 5 min. Sucrose was then added to the postnuclear supernatant with constant stirring and the final volume was adjusted to the sucrose concentration to 40% (W/V). The suspension was centrifuged at 48,000 g for 1 h in a Beckman L5-50B ultracentrifuge. The supernatant completely removed and the pellets were resuspended in 0.25 M sucrose. The suspensions were centrifuged at 48,000 g for 1 h and the pellets (granule rich fraction) were suspended in 25% ethylene glycol with a Teflon glass homogenizer (Gabig *et al.*, 1982). The protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of superoxide radical generation

The superoxide dependent reduction of ferricytochrome c was measured by the method of Markert *et al.* (1984). The reaction mixtures in

plastic microfuge tubes contained 10^6 neutrophils, HBSS buffer, 75 μM ferricytochrome c, 50 μM arachidonic acid, 20 mM HEPES-tris, pH 7.4 and other compounds in a total volume of 500 μl. The reactions were performed in a 37°C shaking water bath for 10 min. The reaction was then stopped by placing the tubes in melting ice and the cells were rapidly pelleted by centrifuging at 1,500 g for 5 min at 4°C. The supernatants were taken and the amount of reduced cytochrome c was measured at 550 nm in a Gilford 260 U.V.-spectrophotometer. The amount of reduced cytochrome c was calculated by using an extinction coefficient of 1.85×10^4 $M^{-1}cm^{-1}$ at 550 nm.

Assay of hydrogen peroxide generation

H_2O_2 produced from activated neutrophils was measured by change of scopoletin fluorescence. The reaction mixtures contained 2×10^6 neutrophils, 2.5 μM scopoletin, 5 μg/ml horse radish peroxidase, arachidonic acid and HBSS buffer in a total volume of 2.0 ml. After preincubation of 5 min at 37°C, the reaction was initiated by addition of arachidonic acid. The decrease of scopoletin fluorescence by H_2O_2 produced was read at the wavelength of excitation, 343 nm and emission, 460 nm (Root *et al.*, 1975).

Assay of NADPH oxidase activity

The activity of NADPH oxidase was measured as reduction of ferricytochrome c by superoxide radicals produced from oxidation of NADPH by NADPH oxidase. The reaction mixture consisted of 0.1 mg/ml granule rich fraction, 100 μM NADPH, 75 μM ferricytochrome c, 50 μM arachidonic acid, 20 mM HEPES-tris, pH 7.4 and other compounds in a total volume of 500 μl. The reaction mixture was preincubated for 5 min at 37°C and the reaction was initiated by adding NADPH. The reduction rate of ferricytochrome c was measured at 550 nm (Lee *et al.*, 1987).

Assay of beta glucuronidase

Released β-glucuronidase in reaction mixtures consisted of 2×10^6 neutrophils, 50 μM arachidonic acid, 1 mM phenolphthalein-glucuronic acid, pH 7.0, 60 mM citrate buffer, pH 4.6 and other compounds in a total volume of 500 μl. After 18 h of incubation at 37°C, reaction was stopped by adding 2 ml of 0.2 M ice-cold glycine buffer, in 0.2 M NaCl, pH 10.4 and absorbance was read at 500 nm. β-Glucuronidase activity is expressed as μg phenolphthalein/18 h/2×10^6 cells (Brittinger *et al.*, 1968).

Measurement of calcium release by neutrophils

Calcium release was measured by the spectrophotometric method using an Aminco-Chance dual wavelength-split beam spectrophotometer. The reaction mixtures contained 10^6 cells/ml of neutrophils, 50 μM murexide, HBSS buffer and 20 mM HEPES-tris, pH 7.4. After preincubation at 37°C for 10 min, the reaction was initiated by addition of various concentration of arachidonic acid with 1 mM calcium and a final volume was a 1.0 ml. The rate and extent of calcium release by neutrophils was measured with the absorbance changes of calcium chelating dye, murexide, at 507-540 nm in a 1.0 ml cuvette (Malmstrom and Carafoli, 1979).

Measurement of cytosolic free calcium

Quin 2 loading and fluorescence measurement was performed by the modification of the method of Tsien *et al.* (1982). Neutrophils (approximately 10^8/ml) were loaded with 5 μl of 20 mM quin 2/AM at 37°C for 20 min in 1.0 ml of the reaction mixtures containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM dextrose. The suspension was then diluted to 10 fold with the above reaction mixture and further incubated at 37°C for 40 min. After loading, the suspension was centrifuged at 1,500 g for 5 min and neutrophils were resuspended in the above reaction mixture as approximately 10^7/100 μl.

Fluorescence measurement was done with a Turner Spectrofluorometer (Model/430). Preloaded neutrophils (10^7) were suspended in the same reaction mixture in a final volume of 2.0 ml. After preincubation at 37°C for 5 min, reaction was initiated by addition of arachidonic acid. The fluorescence change was read at an excitation wavelength of 339 nm and emission wavelength of 492 nm.

RESULTS

Superoxide and hydrogen peroxide generation in neutrophils activated by arachidonic acid

Stimulation of respiratory burst by exogenous

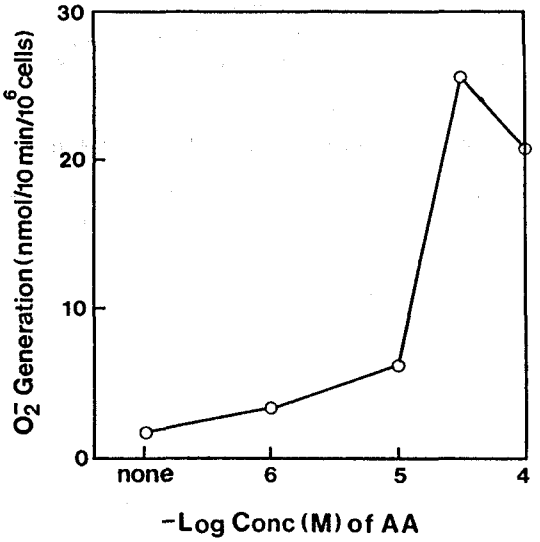

Fig. 1. Stimulation of superoxide generation by arachidonic acid in neutrophils. Neutrophils (10^6 cells/0.5 ml) were incubated with varying concentration of arachidonic acid (AA) for 10 min at 37°C. Points are means of 9 experiments.

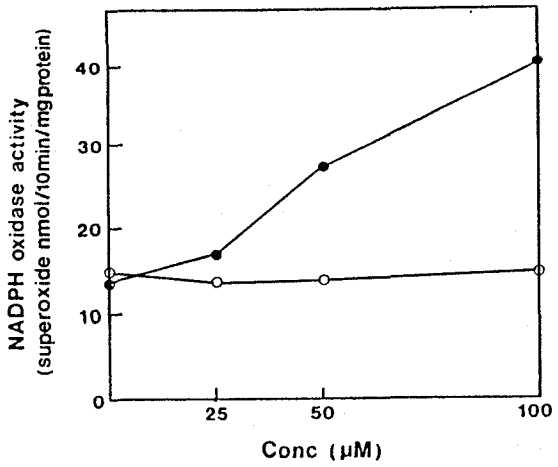

Fig. 3. Effect of arachidonic acid on NADPH oxidase activity in Ca^{++}-free or Ca^{++}-containg reaction medium. The reaction mixtures contained 0.1 mg/ml granule rich fraction, 100 μM NADPH, 75 μM ferricytochrome c, varying concentration of arachidonic acid, 1 mM $CaCl_2$ and 20 mM HEPES-tris, pH 7.4. Ca^{++}-free medium was made by addition of 10 mM EGTA on the above mixture which did not contain exogenously added Ca^{++}. The response was initiated by addition of NADPH and arachidonic acid, simultaneously. Points are means of 3-6 experiments. O in Ca^{++}-free medium, ● in Ca^{++} containing medium.

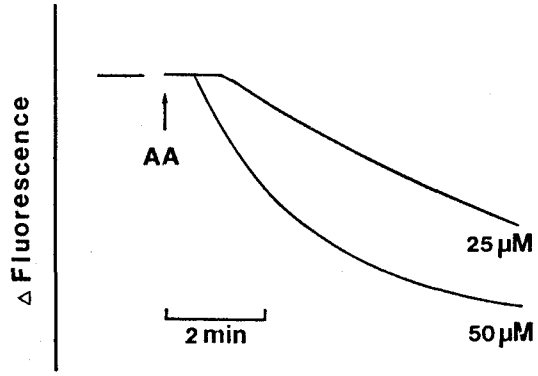

Fig. 2. Stimulation of hydrogen peroxide generation by arachidonic acid in neutrophils. Two ml of reaction mixtures contained 2×10^6 neutrophils, 2.5 μM scopoletin, 5 μg/ml horse radish peroxidase, arachidonic acid and HBSS buffer. After preincubation of 5 min, the response was initiated by addition of arachidonic acid (AA). Fluorescence change of scopoletin was measured fluorometrically at the wavelength pair 343-460 nm.

arachidonic acid was investigated. Fig. 1 showed that exogenous arachidonic acid stimulated superoxide generation in intact neutrophils in a dose dependent fashion. Amount of superoxide generated in neutrophils activated by 50 μM arachidonic acid was 25.53 nmol/10 min/10^6 cells. H_2O_2 produced, which is attained from the dismutation of O_2^- (Park *et al.*, 1987), was measured by the decrease of scopoletin fluorescence. Oxidation of scopoletin in neutrophils was stimulated with arachidonic acid (Fig. 2).

Necessity of Ca^{++} in activation of NADPH oxidase by arachidonic acid was observed. As shown in Fig. 3, in Ca^{++} free medium, stimulatory effect of arachidonic acid on NADPH oxidase which is obtained from resting neutrophils was not detected up to 100 μM. However, in Ca^{++} containing medium arachidonic acid markedly activated NADPH oxidase and at 50 μM, oxidase activity was increased to about 2.1 times.

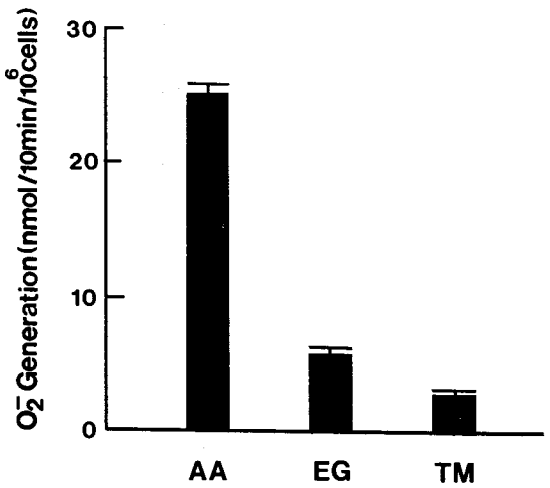

Fig. 4. Inhibitory effects of EGTA and TMB-8 on superoxide generation stimulated by arachidonic acid. Neutrophils (10^6 cells/0.5 ml) were incubated with 50 μM arachidonic acid in the presence of either 10 mM EGTA or 0.5 mM TMB-8. Values are means±SEM of 9 experiments. AA, arachidonic acid; EG, EGTA and TM, TMB-8 in the presence of arachidonic acid.

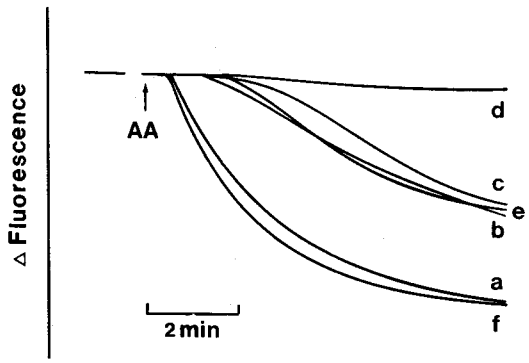

Fig. 5. Effects of compounds on hydrogen peroxide generation stimulated by arachidonic acid. Neutrophils (2×10^6 cells/2 ml) were preincubated with compounds for 5 min and the response was initiated by addition of arachidonic acid (AA). Fluorescence change of scopoletin was measured fluorometrically at the wavelength pair 343-460 nm. a, none; b, 10 mM EGTA; c, 0.5 mM verapamil; d, 100 μM dibucaine; e, 10 μM CCCP; f, 0.5 mM theophylline in the presence of 50 μM arachidonic acid.

Table 1. Effects of ionic channel blockers on arachidonic acid-stimulated superoxide generation in neutrophils

Compounds		Superoxide nmol/10 min /10^6 cells
Arachidonic acid	50 μM	25.75±0.62
+ Verapamil	500 μM	3.61±0.16
	100 μM	20.31±0.55
+ Diltiazem	100 μM	16.80±0.88
+ Nifedipine	100 μM	11.45±1.92
+ TTX	10 μM	28.09±0.50
+ TEA	100 μM	25.59±1.03

Neutrophils (10^6 cells/0.5 ml) were preincubated with compounds in HBSS for 5 min and the response was initiated by addition of arachidonic acid. Values are means± SEM of 3-9 experiments.

Arachidonic acid-stimulated superoxide and H_2O_2 generation was inhibited by EGTA, a calcium specific chelator and TMB-8, an intracellular calcium chelator (Fig. 4 and Fig. 5).

These findings indicate that extra-and intra-cellular calcium may be required for the stimulatory effect of arachidonic acid on intact neutrophils.

Effects of ionic channel blockers, local anesthetics, sulfhydryl (SH) group inhibitors and membrane depolarization suppressants on arachidonic acid-stimulated respiratory burst

Activated neutrophils result in molecular changes, including Na^+ influx and Ca^{++} mobilization. Although it is demonstrated that arachidonic acid significantly elevates cytosolic free Ca^{++} level, effect of arachidonic acid on Ca^{++} influx and efflux in neutrophils is unknown. Thus, effects of ionic channel blockers on superoxide generation by arachidonic acid were examined. Fifty μM arachidonic acid-stimulated superoxide generation was inhibited by 100 μM of Ca^{++} channel blockers verapamil, diltiazem and nifedipine but not 10 μM tetrodotoxin, a Na^+ channel blocker and 100 μM tetraethylammonium chloride, a K^+ channel blocker (Table 1).

Local anesthetics appear to block nerve conduction by their effect on membrane fluid partly (Tayler and Insel, 1990). Table 2 showed that local anesthetics, dibucaine and lidocaine inhibited stimulatory action of arachidonic acid, but pro-

Table 2. Effects of local anesthetics on arachidonic acid-stimulated superoxide generation in neutrophils

Compounds		Superoxide nmol/10 min /10^6 cells
Arachidonic acid	50 μM	25.62±0.63
+ Dibucaine	1 mM	3.50±0.23
	0.1 mM	4.90±0.43
+ Lidocaine	1 mM	19.31±2.00
	0.1 mM	21.79±2.21
+ Procaine	1 mM	24.44±0.16
	0.1 mM	25.86±0.49

Neutrophils (10^6 cells/0.5 ml) were preincubated with compounds in HBSS for 5 min and the response was initiated by addition of arachidonic acid. Values are means± SEM of 3-6 experiments.

Table 3. Inhibition of arachidonic acid-stimulated superoxide generation by phosphorylation inhibitors of membrane protein and chlorpromazine

Compounds		Superoxide nmol/10 min /10^6 cells
Arachidonic acid	50 μM	25.58±0.87
+ CCCP	10 μM	8.90±0.38
+ 2,4-Dinitrophenol	100 μM	11.14±1.32
+ Sodium arsenate	100 μM	11.65±0.43
+ Chlorpromazine	10 μM	14.99±1.33
+ Theophylline	500 μM	16.03±0.77

Neutrophils (10^6 cells/0.5 ml) were preincubated with compounds in HBSS for 5 min and the response was initiated by addition of arachidonic acid. Values are means± SEM of 4-9 experiments.

caine did not affect it.

The activation of receptors for neurotransmitters is thought to initiate cellular responses by opening the specific Ca^{++} channels in the cell membrane through membrane depolarization and protein phosphorylation (Lew *et al.*, 1984: Pontremoli *et al.*, 1986). Preveious work showes that CCCP significantly inhibits ATP-stimulated superoxide generation in neutrophils activated by opsonized zymosan (Park and Lee, 1990). Table 3 showed that 10 μM CCCP, 100 μM 2,4-DNP and 100 μM sodium arsenate inhibited 50 μM arachidonic acid-stimulated superoxide generation.

Table 4. Effects of various compounds on arachidonic acid-activated NADPH oxidase activity

Compounds		NADPH oxidase activity Superoxide nmol/10 min/mg protein
Arachidonic acid	50 μM	27.56±0.99
+ Dibucaine	100 μM	18.13±1.04
+ CCCP	10 μM	16.98±1.00
+ Chlorpromazine	10 μM	16.02±0.92
+ Theophylline	500 μM	22.26±0.92

NADPH oxidase activity in the absence of arachidonic acid was 12.97±0.27 nmol/10 min/mg protein of superoxide ion. NADPH oxidase containing granule rich fraction was preincubated for 5 min and the response was initiated by addition of NADPH and arachidonic acid, simultaneously. Values are means±SEM of 3-4 experiments.

On the other hand, stimulatory action of arachidonic acid on superoxide generation was inhibited by chlorpromazine, an inhibitor of protein kinase C and calmodulin and theophylline, an inhibitor of cyclic nucleotide phosphodiesterase.

H_2O_2 generation in the presence of 50 μM arachidonic acid was markedly inhibited by 10 mM EGTA, 0.5 mM verapamil, 100 μM dibucaine and 10 μM CCCP (Fig. 5). 0.5 mM Theophylline had no any significant effect.

In Ca^{++} containing medium, stimulatory action of arachidonic acid on NADPH oxidase was inhibited by dibucaine, CCCP and chlorpromazine and slightly inhibited by theophylline (Table 4).

Since the SH groups of the plasma membrane and an intracellular soluble thiols are suggested to be prerequisite for the expression of activated neutrophil's response (Voetman *et al.*, 1980), effects of a cell penetrable SH group inhibitor, $HgCl_2$ and surface SH group inhibitors, PCMB and PCMBSA on arachidonic acid-stimulated superoxide generation were investigated. As can be seen in Table 5, 50 μM arachidonic acid-stimulated superoxide generation was effectively interfered with 10 μM $HgCl_2$, 100 μM PCMB and 100 μM PCMBSA.

Effects of EGTA, verapamil and other compounds on β-glucuronidase release from activated neutrophils

After neutrophils were preincubated for 10 min with various compounds, β-glucuronidase release

Table 5. Inhibition of arachidonic acid-stimulated superoxide generation by sulfhydryl group inhibitors in neutrophils

Compounds	Superoxide nmol/10 min /10^6 cells	
Arachidonic acid	50 μM	25.49±0.93
+ $HgCl_2$	10 μM	4.54±0.89
+ PCMB	100 μM	15.96±0.56
+ PCMBSA	100 μM	17.14±1.56

Neutrophils (10^6 cells/0.5 ml) were preincubated with compounds for 5 min and the response was initiated by addition of arachidonic acid. Values are means±SEM of 3-6 experiments.

Table 6. Effects of various compounds on β-glucuronidase release from arachidonic acid-stimulated neutrophils

Compounds	μg Phenolphthalein / 18 h/2×10^6 cells	
Arachidonic acid	50 μM	29.53±0.62
+ EGTA	10 mM	1.26±0.41
+ Verapamil	0.5 mM	23.28±0.64
+ Dibucaine	100 μM	27.92±0.52
+ CCCP	10 μM	21.72±1.13
+ Theophylline	0.5 mM	24.44±0.94

β-Glucuronidase activity of resting neutrophils was 0.73±0.03 μg phenolphthalein/18h/2×10^6 cells. β-Glucuronidase release from neutrophils was initiated by addition of arachidonic acid in the presence of compounds. Values are means±SEM of 3-5 experiments.

was initiated by arachidonic acid. As shown in Table 6, β-glucuronidase release was almost completely inhibited by EGTA and slightly inhibited by verapamil, CCCP and theophylline. In this reaction, 100 μM dibucaine did not show any significant effect.

Effects of various compounds on arachidonic acid-induced calcium mobilization

Although is well known that arachidonic acid induces Ca^{++} release from liver microsomes and permeabilized neutrophils, it's effect on Ca^{++} influx or efflux in intact neutrophils is still not elucidated. On the other hand, it is reported that arachidonic acid decreases the initial rate of Ca^{++} uptake in rat anterior pituitary cells (Knepel *et al.*, 1988). The result represented in Fig. 6 indicated that arachidonic acid induced Ca^{++} release rather than uptake. TMB-8 decreased amount of Ca^{++} released by arachidonic acid.

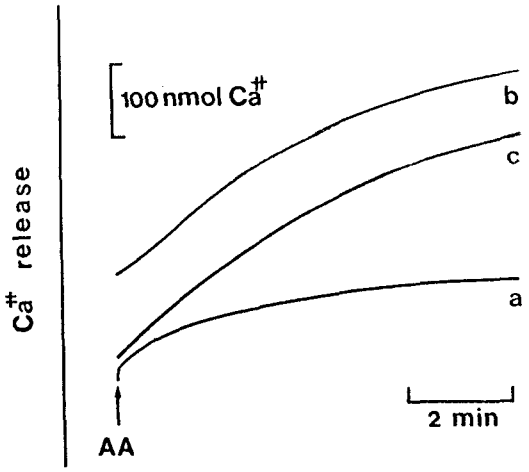

Fig. 6. Arachidonic acid-induced calcium release from neutrophils. The reaction mixtures contained 10^6 cells/ml neutrophils, 50 μM murexide, HBSS and 20 mM HEPES-tris, pH 7.4. After preincubation with or without 0.5 mM TMB-8, the release was initiated by addition of arachidonic acid (AA). Calcium release was measured spectrophotometrically at the wavelength pair 507-540 nm. a, 25 μM AA; b, 50 μM AA; c, 50 μM AA+0.5 mM TMB-8.

The intracellular free Ca^{++} level was measured with quin 2/AM. When neutrophils were exposed to arachidonic acid, fluorescence change due to the complex formation of an increased Ca^{++} with quin 2 occured and at 1.5 min of incubation, fluorescence was increased to a peak level (Fig. 7). The increase in intracellular free Ca^{++} level elicited by 50 μM arachidonic acid inhibited by 10 mM EGTA, 10 μM CCCP and slightly inhibited by 0.5 mM TMB-8. In the presence of arachidonic acid, 0.5 mM verapamil or 10 μM dibucaine steadily elevated intracellular free Ca^{++} level (Fig. 7 and 8). Amount of intracellular free Ca^{++} increased by either arachidonic acid plus verapamil or arachidonic acid plus dibucaine was greater than that by arachidonic acid alone.

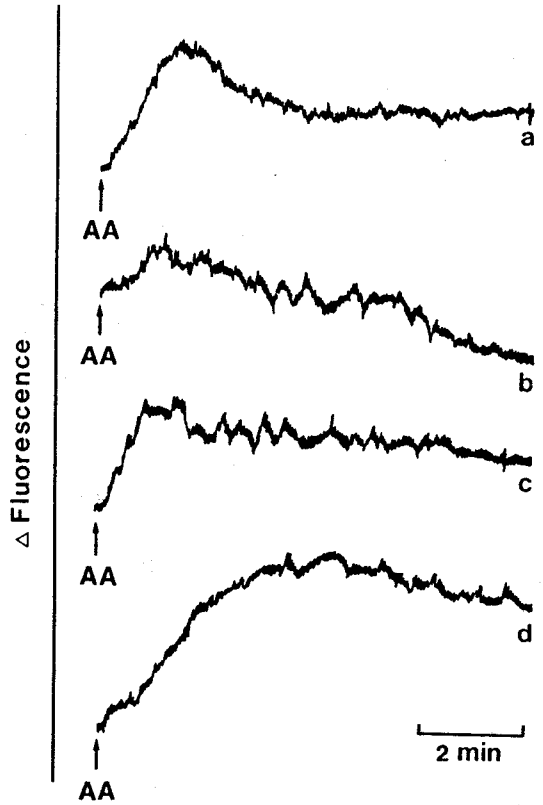

Fig. 7. Effects of EGTA, TMB-8 and verapamil on enhanced cytosolic free calcium level by arachidonic acid in neutrophils. Intracellular free calcium level in arachidonic acid stimulated neutrophils (5×10^6 cells/ml) was measured as a fluorescence change of quin 2, a specific Ca^{++} chelator. Experimental conditions were the same as described in Materials and Methods. The response was initiated by addition of arachidonic acid (AA) in the presence of compounds. Fluorescence of quin 2-Ca^{++} complex was read at the wavelength pair 390-492 nm. a, none; b, 10 mM EGTA; c, 0.5 mM TMB-8; d, 0.5 mM verapamil in the presence of 50 μM arachidonic acid.

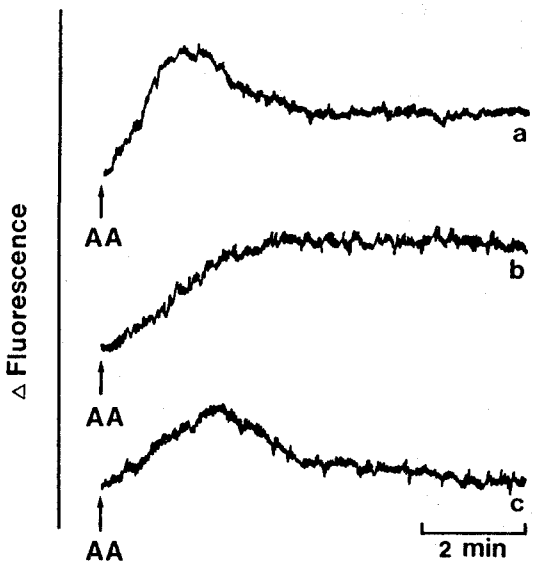

Fig. 8. Effects of dibucaine and CCCP on enhanced cytosolic free calcium level by arachidonic acid. Neutrophils (5×10^6 cells/ml) were preincubated with 10 μM dibucaine and 10 μM CCCP and the response was initiated by addition of arachidonic acid (AA). a, none; b, 10 μM dibucaine; c, 10 μM CCCP in the presence of 50 μM arachidonic acid.

DISCUSSION

Arachidonic acid and other cis-unsaturated fatty acids stimulate the respiratory burst in phagocytic cells (Badwey *et al.*, 1981: Tauber, 1987). Moreover, arachidonic acid has been reported to activate superoxide generation in cell free systems from human neutrophils and guinea pig peritoneal macrophages (Bromberg and Pick, 1984: Curnutte, 1985). Arachidonic acid-stimulated superoxide generation is not affected by inhibitors of lipoxygenase and cyclooxygenase (Badway *et al.*, 1981). Thus, arachidonic acid itself may stimulate superoxide generation in neutrophils, directly. The mechanism by which arachidonic acid activates the superoxide forming NADPH oxidase system in either intact neutrophils or disrupted cell fractions is uncertain. Free fatty acids readily intercalate into the plasma membranes of cells and affect lipid-protein interactions which could bring about functional alterations.

Arachidonic acid is suggested to activate superoxide generation by changing membrane fluidity in the locale of NADPH oxidase (Badwey *et al.*, 1984). It is postulated that arachidonic acid and sodium dodecyl sulfate activate NADPH oxidase by their detergent properties (Bromberg and Pick,

1985). However, Chan and Turk (1987) report that arachidonic acid does not appear to act as a Ca^{++} ionophore or by a detergent-like action to disrupt the integrity of microsomal membranes and increase their permeability in rat liver microsomes. Furthermore, after the interaction of arachidonic acid with the plasma membrane of neutrophils, influences of the followed biochemical events in the activated respiratory burst are still not clarified.

Ca^{++} may be prerequisite for the activation of NADPH oxidase activity, because in Ca^{++} free medium the stimulatory effect of arachidonic acid on NADPH oxidase which is obtained from resting neutrophils was not detected. In addition, arachidonic acid-stimulated superoxide and H_2O_2 generation was inhibited by EGTA, a Ca^{++} specific chelator, TMB-8, an intracellular Ca^{++} chelator, Ca^{++} channel blockers verapamil, diltiazem and nifedipine and calmodulin inhibitors dibucaine and chlorpromazine. Thus, these findings indicate that extra- and intra- cellular Ca^{++} may be required to the expression of functional response of neutrophils activated by arachidonic acid. Arachidonic acid increases K^+ conductance in smooth muscle cells (Ordway *et al.*, 1989) and it is well known that in activated neutrophils Na^+ influx is followed. However, activation of Na^+ and K^+ channel may have a minor effect on superoxide generation in arachidonic acid-activated neutrophils. Local anesthetics appear to block nerve conduction by effect on membrane fluidity change as well as inhibition of Na^+ influx during cell activation (Tayler and Insel, 1990). Dibucaine is also known as a calmodulin inhibitor. Arachidonic acid-stimulated respiratory burst was markedly inhibited by dibucaine and inhibited by lidocaine. Although procaine has no effect, neutrophil's response may be partly attributed to the alteration of membrane fluidity. Activated neutrophils result in membrane depolarization and phosphorylation of membrane protein (Mottola and Romeo, 1982: Pontremoli *et al.*, 1986). Inhibition of the respiratory burst by suppressants of membrane depolarization including CCCP postulates that membrane depolarization by arachidonic acid binding may be also implicated in the expression of neutrophil's response.

The functional responses, such as degranulation of neutrophils can be altered by the change of cytosolic nucleotide level as well as Ca^{++} mobilization. It has been observed that agents which elevate the cAMP level within neutrophils inhibit the release of lysosomal enzymes during feeding of opsonized zymosan, whereas agents which elevate the cGMP level within cells enhance the release of enzymes(Zurier *et al.*, 1974). It is reported that high concentration of arachidonic acid inhibits elevation of cytosolic free Ca^{++} level in platelets by stimulating adenylate cyclase(Kowalska *et al.*, 1988). Degranulation by arachidonic acid was significantly inhibited by EGTA and slightly inhibited by verapamil, CCCP and theophylline. And this finding was similar with their effects on superoxide generation, except dibucaine. Discrepancy of dibucaine effect on neutrophil's response suggests the possibility that the process of the respiratory burst may be different from the degranulation process.

The sulfhydryl groups of plasma membrane and an intracellular soluble thiols are probably prerequisite for neutrophil's response to stimulation, such as phagocytosis(Tsan *et al.*, 1976), degranulation(Wedner *et al.*, 1981) and superoxide generation(Curnutte and Babior, 1975) and subsequent maintenance of function. It is reported that normal GSH levels may be necessary for the transduction of the activation signal from the exterior of neutrophils(Wedner *et al.*, 1981). Previous work showes that during activation, contents of surface and soluble SH groups in neutrophils were gradually decreased with increasing incubation time (Shin *et al.*, 1989). Inhibitions of arachidonic acid-stimulated superoxide generation by a cell penetrable SH group inhibitor, $HgCl_2$(Vansteveninck *et al.*, 1965) and suface acting SH group inhibitors, PCMB and PCMBSA(Tsan *et al.*, 1976) indicate that both arachidonic acid binding to the plasma membrane of neutrophils and the expression of response may be dependent on the existance of reduced SH groups.

Ca^{++} influx and cytosolic Ca^{++} redistribution at neutrophils appear to be regulated by membrane depolarization and receptor binding to the plasma binding(Mottola and Romeo, 1982: Lew *et al.*, 1984). In cardiac muscle, smooth muscle and neural tissues, elevation of the extracellular K^+ concentration leads to membrane depolarization(Bolton, 1979: Cho *et al.*, 1988) and then to the influx of Na^+ and Ca^{++} into the cell which can induce release of transmitter and finally contraction (Crompton *et al.*, 1976: Powis, 1981). On the other hand, many of the neurotransmitters, hormones

and drugs may modulate the efficiency of neurotransmitter release by modifying the permeability for Ca^{++} channels through their effects on cAMP-dependent protein kinases and subsequent phosphorylation in specific proteins at the plasma membrane(Reuter, 1983). Previous experiment observes that atropine, phentolamine and propranolol interfere with Ca^{++} influx and inhibit superoxide generation in opsonized zymosan-activated neutrophils(Lee *et al.*, 1988). ATP and adenosine may further alter functional responses of opsonized zymosan-activated neutrophils by increasing Ca^{++} uptake and cytosolic free Ca^{++} level(Park and Lee, 1990). However, in the present study, arachidonic acid rather induced Ca^{++} release from intact neutrophils and it was decreased by TMB-8, an intracellular Ca^{++} chelator. This finding suggests that part of released Ca^{++} may be dervied from intracellular Ca^{++} storage sites. Ca^{++} efflux by arachidonic acid seems to be not attained by effect on the outward Ca^{++} transport system, because arachidonic acid does not affect Ca^{++} ATPase in liver microsomes(Chan and Turk, 1987). This suggession can be supported by the results that intracellular Ca^{++} level in the presence of arachidonic acid plus verapamil is greater than that of arachidonic acid alone. Arachidonic acid-induced elevation of cytosolic free Ca^{++} level is slightly decreased by TMB-8 but further increased by verapamil and dibucaine. The result indicates that arachidonic acid may elevate cytosolic free Ca^{++} level by stimulating Ca^{++} release from intracellular Ca^{++} storage sites. In addition, the inhibitory effect of EDTA also postulates that during activation of neutrophils, Ca^{++} influx and efflux may be accomplished, simultaneously.

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= 국문초록 =

사람 중성호성 백혈구의 기능에 있어서 Arachidonic Acid의 활성화 기전

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칼슘을 함유하는 반응액에서 arachidonic acid는 뚜렷하게 superoxide와 H_2O_2의 생성을 자극하였고 NADPH oxidase를 활성화하였다. 칼슘이 없는 반응액에서 NADPH oxidase에 대한 arachidonic acid의 자극 작용은 나타나지 않았다. Arachidonic acid에 의하여 자극된 respiratory burst는 EGTA, TMB-8, verapamil, diltiazem, nifedipine, dibucaine, lidocaine, CCCP, 2,4-dinitrophenol, sodium arsenate, chlorpromazine, theophylline, $HgCl_2$, PCMB와 PCMBSA에 의하여 억제되었으나, tetrodotoxin, tetraethyl ammonium chloride와 procaine의 영향은 받지 않았다. EGTA는 거의 완전히 arachidonic acid에 의한 β-glucuronidase의 유리를 억제하였으며, verapamil, CCCP와 theophylline은 약간 억제하였으나, 이에 반하여 dibucaine은 유의한 효과를 나타내지 않았다. Arachidonic acid는 중성호성 백혈구로 부터 칼슘을 유리시켰으며, 이는 TMB-8에 의하여 감소되었다. Arachidonic acid에 의한 세포내 유리 칼슘 농도의 상승은 EGTA와 CCCP에 의하여 억제되었고 TMB-8에 의하여 약간 억제되었다. Arachodionic acid와 verapamil 또는 arachidonic acid와 dibucaine의 동시 첨가에 의하여 상승된 세포내 유리 칼슘 농도의 양은 arachidonic acid 단독에 의한 것보다 현저하였다.

이상의 결과로 부터 다양한 생화학적 변화가 arachidonic acid에 의하여 활성화된 중성호성 백혈구의 기능 표현에 관여할 것으로 시사된다. Arachidonic acid는 세포내 칼슘 저장고로부터 칼슘 유리를 자극하여 세포내 유리 칼슘 농도를 상승시킬 것으로 추정된다. 중성 호성 백혈구의 활성화 중에 칼슘 유입과 유출은 동시에 이루어질 것으로 추정된다.