Excess formation of lysophosphatidic acid with age inhibits myristic acid-induced superoxide anion generation in intact human neutrophils

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Abstract A superoxide anion generation rate upon exposure to myristate of 1.93 ± 0.34 nmol/min/10^6 cells in neutrophils from elderly human donors was significantly less than a value of 3.02 ± 0.48 nmol/min/10^6 neutrophils from young donors. Myristate activation resulted in equal increases of AA in both the young and the old indicating no effect of aging on the PLA_2 pathway to response. By contrast, the PLD-induced generation of PA was significantly higher in the old than in the young. In addition, myristate induced a significant age-related enhancement in LPA generation, in the old but not in the young. The mass of LPA generated following activation was 3.5 nmol/2.5×10^7 cells/ml in the young while in the old it averaged 7.0 nmol/2.5×10^7 cells /ml. The inhibitory effects of LPA may explain the age-related impaired ability to generate superoxide anion following activation by myristate.

Key words: Aging; Saturated fatty acid; Oxidative burst; Phospholipase; Phosphatidic acid

1. Introduction

Aging is associated with declines in signal transduction in activated neutrophils, as evidenced by impaired mobilization of intracellular calcium stores, influx of extracellular calcium [1], altered production of phosphoinositides [2], and reductions in DG and IP3 generation [2,3]. Stimulation of neutrophils have been demonstrated to activate multiple signal transduction pathways to generate superoxide anion [4]. Besides chemotactic stimuli, several ligands, including fatty acids, both saturated and unsaturated, have been demonstrated to be potent stimulators of superoxide anion in intact neutrophils and in cell free systems [5–7]. Activation mediated by arachidonic acid, an unsaturated fatty acid, has been the focus of several studies [6]. However, the pathways of neutrophil stimulation by saturated fatty acids such as myristic acid remain poorly defined. This study examined the effects of age on myristate-induced activation of neutrophils. We show a significant age-related decrease in the rate of superoxide generation in neutrophils upon treatment with myristate. We also obtained insights into the mechanism by which myristate induces superoxide generation in neutrophils.

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Abbreviations: PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; LPA, lysophosphatidic acid; DG, diacylglycerol; IP_3, inositol trisphosphate; TLC, thin layer chromatography; FMLP, *n*-formyl-methionyl-leucyl-phenylalanine; AA, arachidonic acid

2. Materials and methods

2.1. Reagents

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) except Dextran T70 (Pharmacia, Uppsala, Sweden), ionomycin (Calbiochem, La Jolla, CA), [^{3}H]arachidonic acid (100 Ci/mmol), [^{32}P]phosphoric acid (DuPont NEN, Boston, MA), and absolute ethanol (Midwest Grain Products, Co., Pekin, IL).

2.2. Neutrophils

Venous blood from healthy volunteers was drawn in acid-citrate-dextrose solution. All young (ages 21–30) and old (ages 65–75) volunteers were screened to exclude the presence of disease or medications that could have any influence on neutrophil functions, only healthy donors who were on no medication during the course of the study were recruited. Neutrophils were separated by using dextran sedimentation and Ficoll density gradient followed by water lysis of erythrocytes as previously described [8].

2.3. Superoxide generation

Generation of superoxide anion in neutrophils was measured by continuous reduction of ferricytochrome *c* as previously described [8]. Myristic acid was dissolved in ethanol and dried under nitrogen gas. To make sodium salts, they were neutralized in 1 M sodium hydroxide and appropriately adjusted to pH 7.2. Sodium salts of myristate were sonicated at 30% power using a sonic dismembrator model 300 (Fisher Scientific Co., Pittsburgh, PA) just before use to ensure dispersion in the buffer. Neutrophils were preincubated in the presence or absence of 20 μM LPA [9] at 37°C for 10 min. Cells were stimulated by 500 μM myristic acid [7] in the absence of calcium or magnesium [7,10]. The absorbance at 550 nm was followed by Gilford Response II spectrophotometer (Ciba Corning Diagnostic Corp., Oberlin, OH). The effects of preincubation were calculated as percent generation of superoxide when compared to control values.

2.4. Phospholipase A_2 activity

The activity of PLA_2 was determined by measuring release of [^{3}H]arachidonic acid from [^{3}H]arachidonic acid labeled neutrophils as previously described [11]. Cells were stimulated with 500 μM myristic acid in the absence of calcium or magnesium [7,10] or 1 μg/ml ionomycin in calcium and magnesium containing PBS for 15 min at 37°C. This time point was chosen because no significant increase of radioactivity was found with shorter periods of stimulation. The samples of supernatants were counted for radioactivity by 2200 CA TRI-CARB liquid scintillation analyzer (Packard Instruments Co., Grove, IL).

2.5. Formation of PA and LPA

Neutrophils (2.5×10^7 cells/ml) were incubated with [^{32}P]orthophosphoric acid (0.25 mCi/ml) at 37°C for 60 min. Formation of PA and LPA was examined by using thin layer chromatography (TLC) technique as previously described [12]. The radioactivity corresponding to PA and LPA on the TLC plates was analyzed by a PhosphorImager using the program ImageQuant from Molecular Dynamics (Sunnyvale, CA). Data are expressed as relative change of radioactivity in stimulated neutrophils when compared to respective unstimulated controls. Mass of LPA generated per million neutrophils was determined by assessing total incorporation into phospholipids and the amount of LPA generated per assay and is represented as nmol/2.5×10^7 cells/ml.

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2.6. Statistical analysis

Data are expressed as means ± standard error of the mean (S.E.M.). Data were analyzed using Student's *t*-test, values of $P < 0.05$ were considered to be significant.

3. Results and discussion

Treatment with myristic acid resulted in a superoxide generation rate of 3.02 ± 0.48 nmol/min/10^6 neutrophils from young volunteers. This was significantly higher than the value of 1.93 ± 0.34 nmol/min/10^6 cells in neutrophils from old volunteers ($P < 0.05$, Fig. 1). Although the rate was compromised, total superoxide eventually generated in 10 min was the same in the young and the old (Fig. 1). As the rate of superoxide generation is likely to have an effect in the mediation of neutrophil function in vivo, this finding of age-associated alteration in the rate is of significance. Therefore, we characterized the signal transduction pathway employed by myristate in the activation of superoxide in the neutrophil.

We first analyzed PLA_2 activity following activation with myristate. For this purpose, we measured the generation of AA, a metabolite generated by the action of PLA_2. When [^{3}H]AA labeled neutrophils were stimulated by myristic acid, [^{3}H]AA released by neutrophils from young volunteers was 89.89 ± 7.67 cpm, significantly higher than the control value of 62.87 ± 7.67 cpm in unstimulated cells ($P < 0.05$). Similar increases were noted in old volunteers, averaging 84.50 ± 11.29 cpm following activation compared with 62.72 ± 7.39 cpm in the unstimulated cells (Fig. 2). Similar increases were noted in the young and the old following exposure to ionomycin, a potent stimulator of AA production in neutrophils [13]. It should, however, be pointed out that the measurement of AA was carried out only at 15 min following activation, unlike the superoxide generation, which was assayed at earlier time points. Therefore, the AA generation profile may not represent the induced superoxide profile detected in our assays. Thus, alterations in AA production cannot explain the impaired rate of superoxide generation upon activation by myristate. In our hands detectable AA could only be measured at 15 min following activation, hence we have presented the data obtained at this time point.

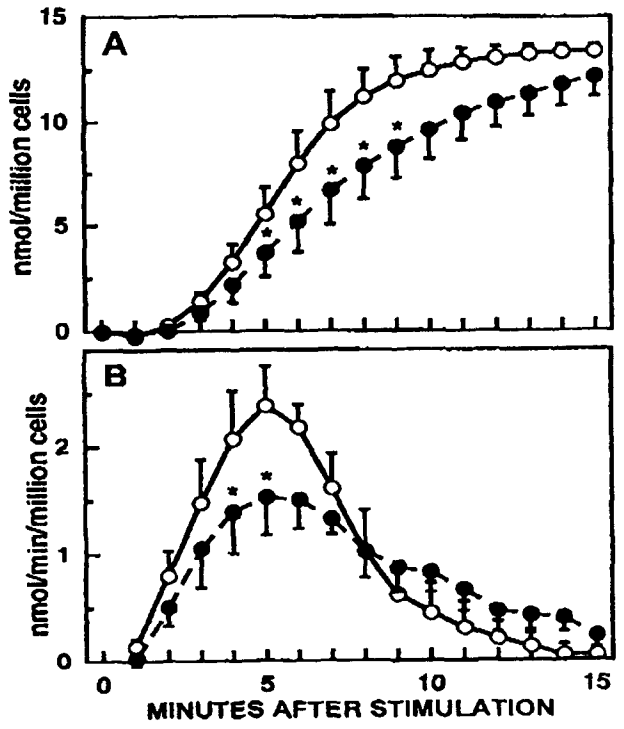

Fig. 1. Superoxide anion generation by intact human neutrophils after exposure to 500 μM myristic acid. Neutrophils were obtained from 6 young (○ and □) and 6 old (● and ■) volunteers. The total amount (A) and the rate of generation (B) were calculated per minute. The asterisks indicate the values from old volunteers were significantly lower than the values from young volunteers ($P < 0.05$).

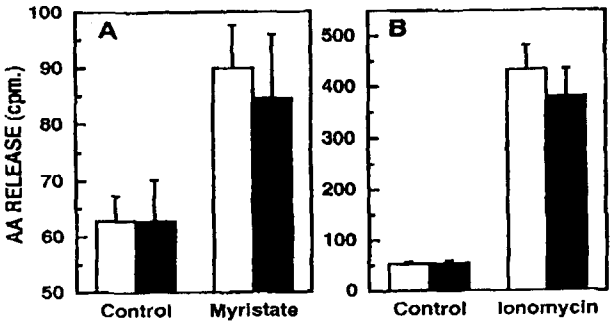

Fig. 2. PLA_2 activity in neutrophils from 6 young (□) and 6 old (■) volunteers. The values are expressed as counts per minute (cpm) in supernatant of each sample. Myristate was used in the absence (A) and presence (B) of calcium. No significant difference was noted between young and old samples.

We next examined the effects of myristate on the PLD pathway to response. The generation of PA in neutrophils from young volunteers 4 min after the exposure to myristic acid averaged 139.8 ± 7.7% of control and was significantly lower than the value of 260.9 ± 29.27% of control observed in neutrophils from the elderly ($P < 0.01$, Fig. 3). The increased generation of PA following activation by myristate in neutrophils from old volunteers suggest no decrements in function of this pathway to cellular response. It also shows that alterations in PA production cannot explain the age-related impaired responsiveness to myristate. We have recently shown a greater age-related dependence on the PLD pathway to response in neutrophils [14]. Thus, following stimulation with the chemotactic peptide FMLP, PA production is not significantly reduced in neutrophils obtained from old donors. This contrasts with the age-related impaired ability to increase cytosolic calcium concentration and to generate IP_3 and DG [1,2]. Increased dependence on the PLD pathway may explain the increased generation of PA following myristate induced activation in neutrophils from old volunteers.

Recently, attention has been focussed on the possible role of inhibitory factors in the regulation of superoxide generation in neutrophils [9]. In this regard LPA may be particularly important. There is evidence that LPA is an important mediator of oxidant injury in other cell systems [15] and as an inhibitor of a p21rac-GTPase activating protein [16]. p21rac is a member of the ras superfamily of small molecular weight GTP-binding proteins, which has been reported to be required for NADPH oxidase activity [17,18]. Studies have also shown that LPA production acts to inhibit superoxide generation in neutrophils [9]. In this study, we demonstrate a significant increase in myristate-induced generation of LPA in intact neutrophils from old donors. Four min after the addition of myr-

istic acid a highly significant increase in LPA was noted in neutrophils obtained from old volunteers, averaging 234.7 ± 42.1% ($P < 0.05$). By contrast, no significant elevation of LPA was noted in the young, the value averaged 114.6 ± 11.6% 4 min after activation by myristate. Both PA and LPA levels decreased 8 min following stimulation and approached levels similar to that observed in the young (Fig. 3). PLD has been reported to be a major generator of PA [19], catalyzing the hydrolytic cleavage of the terminal phosphate diester bond of glycerophospholipids to PA and the corresponding water-soluble head group [20]. Once PA is formed, PLA_2 has the ability to hydrolyze PA and other *sn*-2 fatty acyl bond of phospholipids to primarily produce arachidonic acid and lysophospholipids [20]. PA has been reported to stimulate neutrophils to generate superoxide [21]. As indicated above LPA has been demonstrated to suppress oxidative burst of neutrophils stimulated by FMLP or phorbol myristate acetate [9]. In the light of these findings it can be postulated that PA and arachidonic acid are the major mediators in the myristate-induced activation pathway of neutrophils leading to the generation of superoxide. In cells from the elderly, activation is associated with increased production of PA which in turn leads to increased generation of LPA. In contrast to the old, very little or no LPA is generated following myristate-induced activation in cells from the young. This may be related to the lower concentrations of PA generated which may be below the K_m of phospholipase A_2.

It seems highly likely that increased concentration of LPA exerts inhibitory effects leading to decreased rate of superoxide generation in neutrophils from old volunteers. To confirm that LPA did indeed inhibit superoxide generation, we pretreated neutrophils with LPA and then activated them with myristate. In both, young and old, a marked inhibition of superoxide generation by LPA pretreatment was observed. Generation rate averaged 45.6 ± 11% of control in the young and 39.03 ± 4.2% of control in the old. The percent inhibition observed by preincubation with LPA approximates that observed in the cells from the elderly. As the concentration of LPA used exogenously was 20 nmoles/ml in solution, and assuming uptake and incorporation of 50% as LPA, the inhibition observed by the exogenous addition approaches levels induced in cells from the elderly upon activation with myristate.

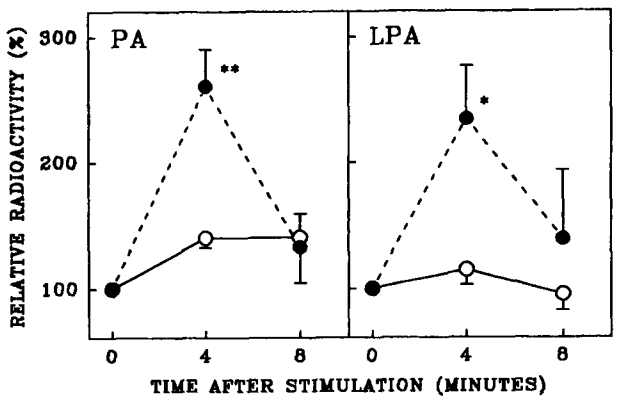

Fig. 3. Formation of PA and LPA in myristic acid-activated neutrophils from 6 young (○) and 6 old (●) volunteers. The asterisks indicate that the values from old volunteers were significantly higher than the values from young volunteers ($^*P < 0.05$ and $^{**}P < 0.01$).

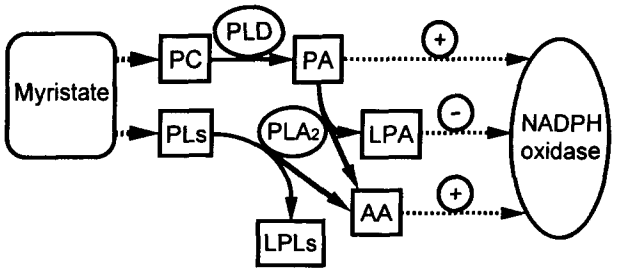

Fig. 4. Proposed mechanism for myristate-induced pathway to superoxide generation. Myristate induces the hydrolysis of phosphatidylcholine (PC) leading to the generation of phosphatidic acid (PA) by the action of PLD. PA when generated in excess is converted to LPA and AA. PA and AA enhance while LPA inhibits superoxide generation. In neutrophils from older persons, increased generation of LPA results in decreased superoxide generation. → indicates mediators generated, + indicates that the mediator has a positive effect on NADPH oxidase activity, − indicates that mediator has a negative effect.

Thus, these results provide new insights into the mechanism of fatty acid-induced stimulation of neutrophils. Our results indicate that myristate-induced superoxide generation is primarily mediated by the activation of PLA and PLD pathway to response. In neutrophils obtained from old volunteers, we suggest that increased dependence on the PLD pathway to cellular response leads to increased generation of PA which in turn leads to increased accumulation of LPA, an inhibitor of superoxide generation. A possible mechanism leading to this inhibition is illustrated in Fig. 4. This can explain the age-related impaired ability to generate superoxide following stimulation by myristate. We believe that the AA in our system, detected at 15 min. occurs much later to impact on the superoxide generation which we have observed. Furthermore, AA generated following 15 min may have been derived from phospholipids such as phosphatidylcholine. This study provides one of the first observations highlighting the importance of considering inhibitory factors as an explanation of declines in neutrophil function that accompany the aging process.

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References

[1] Lipschitz, D.A., Udupa, K.B. and Boxer, L.A. (1988) Blood 71, 659–665.
[2] Lipschitz, D.A., Udupa, K.B., Indelicato, S.R. and Das, M. (1991) Blood 78, 1347–1354.
[3] Fülöp, T. Jr., Varga, Z., Csongor, J., Fóris, G. and Leövey, J. (1989) FEBS Lett. 245, 249–252.
[4] Lackie, J.M. (1988) J. Cell. Sci. 89, 449–452.
[5] Kakinuma, K. and Minakami, S. (1978) Biochim. Biophys. Acta 538, 50–59.
[6] Badway, J.A., Curnutte, J.T. and Karnovsky, M.L. (1981) J. Biol. Chem. 256, 12640–12643.
[7] Tanaka, T., Kanegasaki, S., Makino, R., Iizuka, T. and Ishimura, Y. (1987) Biochem. Biophys. Res. Commun. 144, 606–612.
[8] Babior, B.M. and Cohen, H.J. (1981) in Leukocyte Function (Cline, M.J., Ed.), pp. 1–38, Churchill Livingstone, New York.
[9] Chettibi, S., Lawrence, A.J., Stevenson, R.D. and Young, J.D. (1994) FEMS Immunol. Med. Microbiol. 8, 271–282.

[10] Dana, R., Malech, H.L. and Levy, R. (1994) Biochem. J. 297, 217–223.
[11] Forehand, J.R., Johnston, R.B. and Bomalaski, J.S. (1993) J. Immunol. 151, 4918–4925.
[12] Gerrard, J.M. and Robinson, P. (1984) Biochim. Biophys. Acta 795, 487–492.
[13] Hardy, S.J., Ferrente, A., Poulos, A., Robinson, B.S. Johnson, D.W. and Murray, A.W. (1994) J. Immunol. 153, 1754–1761.
[14] Das, M., Ponnappan, U. and Lipschitz, D.A. (submitted).
[15] Moolenaar, W.H., Jalink, K., Eichholtz, T., Hordijk, P. L., van der Bend, R., van Blitterswijk, W.J. and van Corven, E. (1994) in Cell Lipids (Hoekstra, D., Ed.), pp. 439–450, Academic Press, San Diego, CA.
[16] Ahmed, S., Lee, J., Kozma, R., Best, A., Monfries, C. and Lim, L. (1993) J. Biol. Chem. 268, 10709–10712.
[17] Knaus, U.G., Heyworth, P.G., Evans, T., Curnutte, J.T. and Bokoch, G.M. (1991) Science 254, 1512–1515.
[18] Knaus, U.G., Heyworth, P.G., Kinsella, B.T., Curnutte, J.T. and Bokoch, G.M. (1992) J. Biol. Chem. 267, 23575–23582.
[19] Agwu, D.E., McPhail, L.C., Sozzani, S., Bass, D.A. and McCail, C.E. (1991) J. Clin. Invest. 88, 531–539.
[20] Cockcroft, S. (1992) Biochim. Biophys. Acta 1113, 135–160.
[21] Mitsuyama, T., Takeshige, K. and Minakami, S. (1993) FEBS Lett. 328, 67–70.