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Phospholipase A_2 modulates respiratory burst developed by neutrophils in patients with rheumatoid arthritis

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Abstract

Activated by bacterial peptides, phorbol esters, calcium ionophores and other agonists, neutrophils (PMNs) release the proinflammatory mediator, arachidonic acid (AA) *via* the intervention of phospholipase A_2 (PLA_2). AA may play an essential role in activation of NADPH-oxidase, which is involved in the generation of superoxide anion by neutrophils. The present study is focused on the involvement of PLA_2 in the respiratory burst developed by PMNs isolated from patients with rheumatoid arthritis (RA). PLA_2 exists in very high levels in diseases such as rheumatoid arthritis and may cause acute inflammatory and proliferative changes in synovial structures. The respiratory burst was evaluated as superoxide anion release, using an amplified chemiluminiscence method. The assays were performed using PMNs untreated or treated with different doses of stimulatory reagents (phorbol 12-myristate-13-acetate (PMA), calcium ionophore (A23187)). Our data suggested that PMA stimulated the production of superoxid anion in a dose-response manner, as compared with A23187, which did not induce a significant release of superoxide anion in PMNs-RA. The exogenous addition of AA significantly amplified the superoxide anion release by PMNs-RA stimulated with PMA and to a lesser extent, by PMNs stimulated with A23187. AA has also reversed the inhibitory effect of arachidonyl-trifluorometylketone and E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)2H-pyran-2-one (BEL) on the superoxide anion release by PMNs-RA. In conclusion, the differential responses to these two agents suggested that different isoforms of PLA_2 were activated by A23187 or PMA, and support the idea that activation of these different PLA_2 served distinct functions of PMNs. Therefore, the inhibition of PLA_2 enzymes might be of great importance in the immunotherapy of rheumatoid arthritis.

Keywords: rheumatoid arthritis - neutrophils - phospholipase A2 - calcium ionophore - phorbol 12-myristate-13-acetate - superoxide anion

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology characterized by local production of immunoglobulins, release of cytokines, activation of complement and infiltration of cells within the rheumatoid synovium [1–3]. Although the cause of RA remains unknown, recent data have been substantial in understanding its pathogenesis. It appears that the local release of chemokines by vari-

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ous cell populations leads to the activation of the endothelium of synovial tissue (ST), adherence of PMNs to vascular endothelium and migration of these cells into the extravascular space, due to gradients of chemotactic factors [4]. Neutrophils are phagocytic cells and play a major role in inflammatory diseases such as RA, through production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and oxidized halogens, which are powerful microbicidal agents. ROS are produced during the respiratory burst, which consists of a series of complex reactions beginning by the assembling of a membrane bound enzyme, NADPH oxidase, which is able to catalyze the reduction of oxygen to superoxide anion [5]. NADPH oxidase is assembled on the plasma membrane and then, during phagocytosis, is internalized in the membrane of phagocytic vacuoles which form intracellular phagolysosomes where superoxide anion initiates the formation of other ROS [6,7]. Respiratory burst is triggered in addition to phagocytosis by chemoattractants and immune complexes [8]. Cell and tissue injury associated with acute and chronic inflammation are due to the toxicity of ROS generated and released by activated phagocytes [9].

It is known that when activated by bacterial peptides, phorbol esters, calcium ionophores and other agonists, PMNs release the proinflammatory mediator, arachidonic acid (AA) via the action of phospholipase A_2 (PLA_2) [10,11]. AA serves as a precursor for the generation of a family of bioactive lipid mediators, known as eicosanoids, that includes prostaglandins, thromboxanes and leukotrienes. Recently, it has been shown that AA, as second messenger, has important functional roles, regulating processes such as activation of protein kinase C [12], mitogen - activated protein kinases [13], mobilization of intracellular calcium and activation of calcium channels, and modulation of the activity of potassium channels [14]. In addition, AA may play an essential role in activation of NADPH-oxidase, which is responsible for the generation of superoxide anion by neutrophils [15-17].

PLA_2 plays a central role in releasing lysophosphatides and free fatty acids (such as arachidonic acid) from membrane phospholipids, thereby initiating the production of eicosanoid mediators that profoundly influence inflammatory reaction. PLA_2, when clustered in joint cavities, may accumulate at very high levels in diseases such as rheumatoid arthritis and may cause an acute inflammatory response and proliferative changes in synovial structures. The synovial fluid from rheumatoid arthritis patients contains multiple forms of PLA_2 [18-21]. So, the inhibition of PLA_2 enzymes could represent a target of therapy strategies in rheumatoid arthritis.

Since some observations supported a role of activated PLA_2 in the functions of neutrophils [22], we were interested in demonstrating that PLA_2 is an enzyme involved in the respiratory burst developed by RA neutrophils. Therefore, the present study was focused on the characterization of the effects of exogenous AA and the involvement of PLA_2 in superoxide anion production by PMNs from patients with RA [23,24], activated with A23187 *in vitro* stimulation or PMA. AA has also reversed the inhibitory effect of arachidonyltrifluorometylketone (AACOCF3), the specific inhibitor of cytosolic Ca^{+2}–dependent PLA_2, or E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)2H-pyran-2-one (BEL), the specific inhibitor of calcium independent PLA_2, on the superoxide anion release by PMNs-RA [25-28].

We report here that PMNs isolated from RA patients (RA-PMNs) might trigger significant oxidative stress especially in the inflamed joint. The results of the study indicate that in neutrophils, PMA and A23187 activate different PLA_2 isoforms which are probably involved in distinct biological functions.

Materials and methods

Reagents

HEPES, gelatin, glucose, PMA, calcium ionophore A23187, lucigenin, $CaCl_2$, eosin, arachidonyltrifluorometylketone (AACOCF3), E-6- (bromomethylene) tetrahydro-3-(1-naphthalenyl) 2H-pyran-2-one (BEL) were purchased from Sigma, St. Louis. Ficoll - Hypaque separation media was obtained from Lymphoprep, Nycomed, Oslo, Norway.

Biological samples

Peripheral blood and synovial fluid (SF) were collected from 10 patients diagnosed with rheumatoid arthritis according to the American Rheumatology Association cri-

teria [29,30]. All patients were previously treated with 10 mg prednisolon equivalent per day, but they received no steroid medication 1 month prior to investigation.

PMNs isolated from peripheral blood of 10 healthy volunteers were used as controls.

SF was collected from knee joints in vacuutainer sterile tubes containing EDTA, and used for neutrophils isolation.

Cell isolation

Peripheral blood and synovial PMNs were isolated using Ficoll-Hypaque density gradient centrifugation. When blood was used, erythrocytes were lysed after isolation by using a 0.83% NH_4Cl and 0.084% $NaHCO_3$ solution [31,32]. PMNs were then resuspended in Hanks' balanced salt solution (HBSS) supplemented with 3mM HEPES and 2% gelatin at pH 7.4. Cells were washed three times with HBSS and resuspended at a final concentration of 2 x 10^6 cells /ml in Mg^{+2} – and Ca^{+2} – free HBSS. Cellular viability, scored by eosin exclusion test, exceeded 94%.

Lucigenin amplified chemiluminiscence.

Evaluation of superoxide anion release was performed according to the method previously described by Kharazmi et al. [33]. 200µl samples (1x10^6 cells/ml) with 25mM lucigenin were tested in the presence of scalar concentrations of PMA or A23187. Controls contained no stimulator. In a second set of experiments, samples were additionally treated with specific inhibitors of PLA_2 isoforms: AACOCF3 or BEL. Chemiluminiscence was measured continuously for 15 min using an LKB-Wallak Chemiluminometer. Data were obtained as electrical pulses, expressed in mV and results were shown as percentages of the superoxide release (%), using the formula:

$O_2^{-\cdot}$ release % = (cellular response with stimulus)/(cellular response without stimulus) x 100.

Statistics

Experimental results were expressed as mean value ± standard deviation (SD) for ten experiments performed with cells isolated from different human normal subjects and RA patients. Data were analyzed using Student`s *t*-test. Differences with $p < 0.05$ were considered statistically significant.

Results

The aim of the present study was to define the role of PLA_2 in the oxidative response developed by PMNs isolated from RA patients, activated *in vitro* by PMA or calcium ionophore A 23187. We have comparatively characterized the superoxide anion release either by blood or by synovial PMNs from RA patients, stimulated with PMA or A23187. In order to see the relationship between PLA_2 activation and superoxide anion production, we have investigated the effect of PLA_2 inhibitors (AACOCF3, BEL) on the oxidative activity developed by RA-PMNs. To define the role of PLA_2 in the respiratory burst, we have investigated the ability of exogenous AA in reversing the inhibition of superoxide anion production, previously induced by PLA_2 inhibitors. Peripheral PMNs isolated from normal subjects were used as controls.

Generation and detection of superoxide anion by RA-PMNs

Human phagocytic cells, such as polymorphonuclear leukocytes (PMN), are readily mobilized to sites of infections and produce superoxide by an activated NADPH-oxidase. Our results show that superoxide anion release (%) by unstimulated synovial PMNs from RA patients is higher ($O_2^{-\cdot}$ release = 215%, $p<0.05$) as compared to the corresponding cellular response developed by peripheral RA-PMNs ($O_2^{-\cdot}$ release = 125%, $p<0.02$). Thus, synovial PMNs show an activated status in RA related with the oxidative response (Fig.1).

The effect of PMA on the oxidative response of PMNs in rheumatoid arthritis

PMA is used as a stimulus for neutrophils because it by-passes the multiple signalling pathways triggered by chemoattractants and phagocytic stimuli which activate different surface receptors. The protein-kinase C and respiratory burst activator PMA are potent stimuli for both extracellular and intracellular oxidase activity, and cause marked changes in the morphology of neutrophils [34,35]. The optimal PMA concentration for superoxide anion release was established by dose - effect experiments

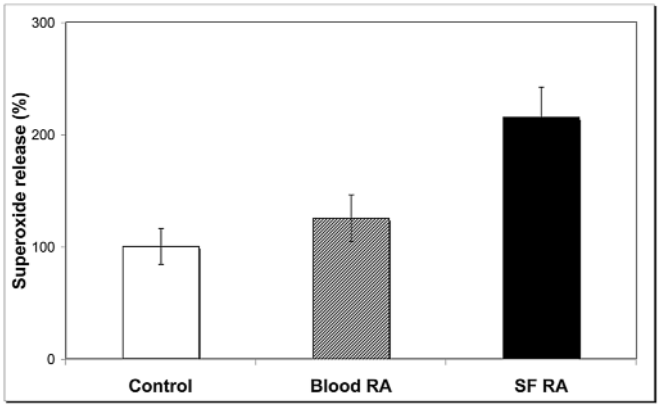


Fig. 1. Superoxide anion release by unstimulated PMNs isolated from peripheral blood or synovial fluid (SF) of RA-patients, as compared to healthy subjects (Control), n=10 in each group. Results are expressed as percentages of superoxide anion release (%).

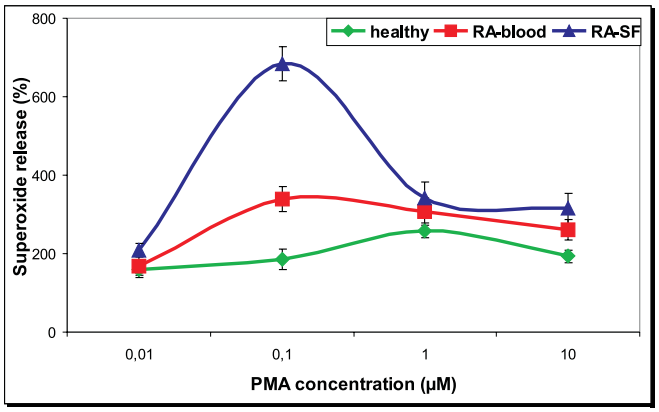


Fig. 2. Effect of PMA concentration on superoxide anion release by PMNs isolated either from peripheral blood or synovial fluid of RA-patients, as compared to healthy subjects (Control), n=10 in each group. Results are expressed as percentages of superoxide anion release (%).

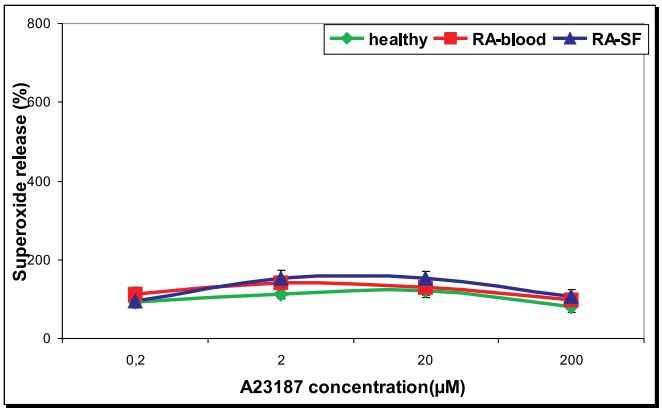
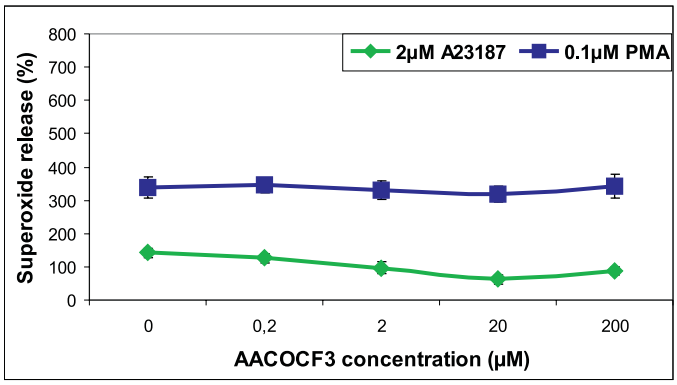


Fig. 3. Effect of calcium ionophore A23187 concentration on the oxidative activity of PMNs isolated either from peripheral blood or synovial fluid of RA-patients, as compared to healthy subjects (Control), n=10 in each group. Results are expressed as percentages of super oxide anion release (%).

performed on PMNs isolated either from peripheral blood of normal and RA patients, or from RA-synovial fluid, treated with scalar concentrations (0.01 µM - 10µM) of PMA (Fig.2).

Our data show that 0.1µM PMA induced the maximum production of superoxide anion by peripheral blood PMNs isolated from normal subjects or RA patients. In RA patients, treating peripheral blood and synovial RA-PMNs with 0.1µM PMA produced a significant response, 2-($p<0.005$) and 4-fold greater respectively ($p<0.001$) than the production of superoxide anion by normal peripheral blood PMNs (Fig. 2). Although synovial PMNs isolated from RA patients are constitutively activated and produce superoxide anion, they respond to PMA stimulation more intensly than peripheral blood PMNs from normals subjects or RA patients.



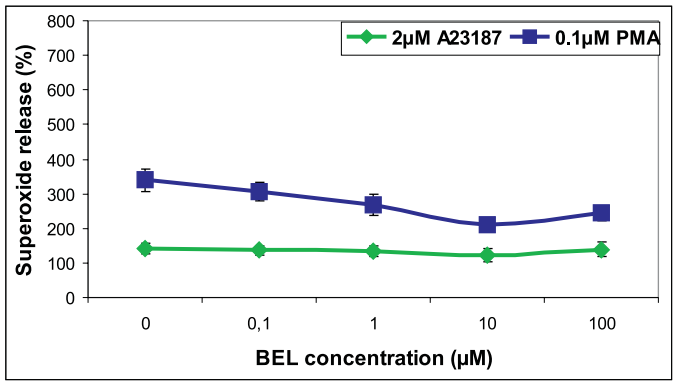


Fig. 4. Inhibition of calcium ionophore or PMA activity on superoxide anion release by AACOCF3 or BEL. Peripheral blood PMNs from RA-patients, were incubated for 15 min in the presence or absence of scalar concentrations of AACOCF3 (panel A) or BEL (panel B), and stimulated for 20 min with 2μM A23187 or 0.1μM PMA. The cellular response developed by RA-PMNs in the absence of stimuli was considered to be 100%, n=10 in each group.

The effect of A23187 on superoxide anion release by rheumatoid arthritis PMNs

We have investigated the ability of A23187 to modulate the superoxide anion generation by PMNs. When A23187 used in concentrations ranging from 0.2μM to 200μM, in the presence of 1mM $CaCl_2$, a reduced superoxide anion production by peripheral blood and synovial RA-PMNs was induced, maximal at 2μM concentration (Fig. 3). Peripheral blood PMNs isolated from normal subjects and stimulated with calcium ionophore did not generate superoxide anion. Absence of $CaCl_2$ from the extracellular medium caused significant reduction in superoxide anion release by A23187-treated neutrophils (data not shown). Statistical analysis show significant differences for superoxide anion release by RA-PMNs from peripheral blood ($p<0.05$) or synovial fluid ($p<0.05$), when compared to the response of control PMNs. Effect of 2μM A23187 on superoxide anion release was similar for peripheral ($O_2^{-\cdot}$ release % = 142%) and synovial PMNs ($O_2^{-\cdot}$ release % = 153%) isolated from RA patients (Fig. 3).

Effects of PLA_2 inhibitors on A23187 or PMA induced superoxide anion production by PMNs

To study the role of PLA_2 in A23187- or PMA -mediated superoxide anion release by RA-PMNs, a set of experiments were performed by treating stimulated cells with scalar concentrations of PLA_2 inhibitors AACOCF3 or BEL. The superoxide anion release by RA-PMNs stimulated with A23187 was inhibited by Ca^{2+}-dependent inhibitor AACOCF3 treatment. The optimal AACOCF3 concentration for inhibiting superoxide anion release was established by dose-effect experiments performed on peripheral blood RA-PMNs. According to our data, the maximum inhibition of superoxide anion production by PMNs was obtained when 20μM AACOCF3 where used (Fig. 4A).

There have been recently described many isoforms of calcium independent-PLA_2, which are inhibited by specific reagents such as bromoenol lactone (BEL) [36-39]. The effect of BEL on the superoxide anion release in rheumatoid arthritis neu-

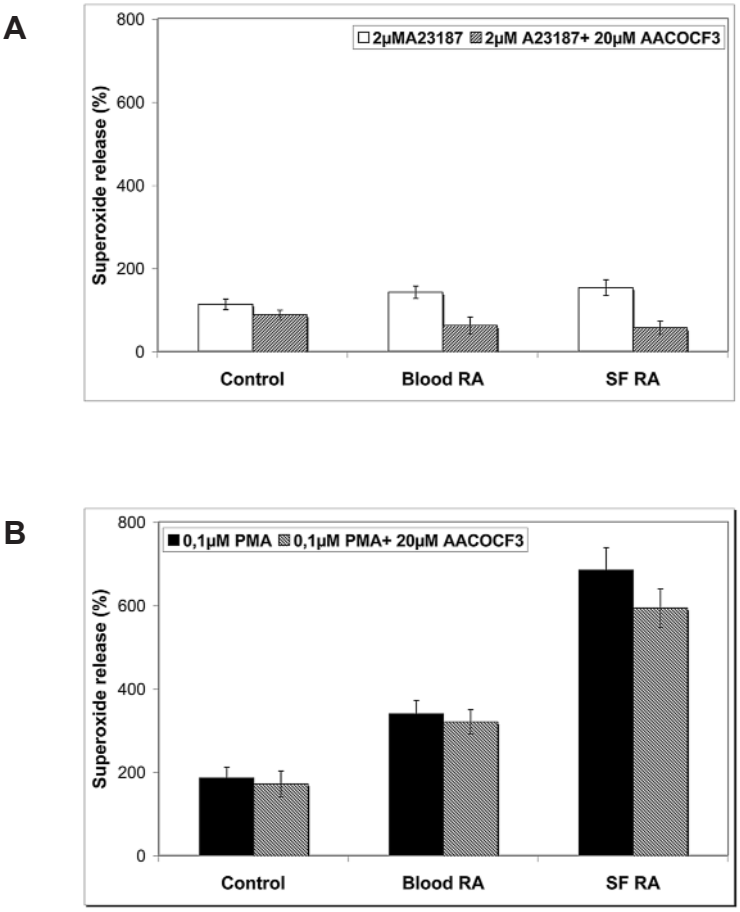


Fig. 5. Effect of 20μM AACOCF3 on superoxide anion release by peripheral blood and SF PMNs from RA patients, activated *in vitro* with 2μM A23187 (A) or 0.1μM PMA (B), as compared to healthy donors. The cellular response developed by PMNs in the absence of stimuli was considered to be 100%, n=10 in each group.

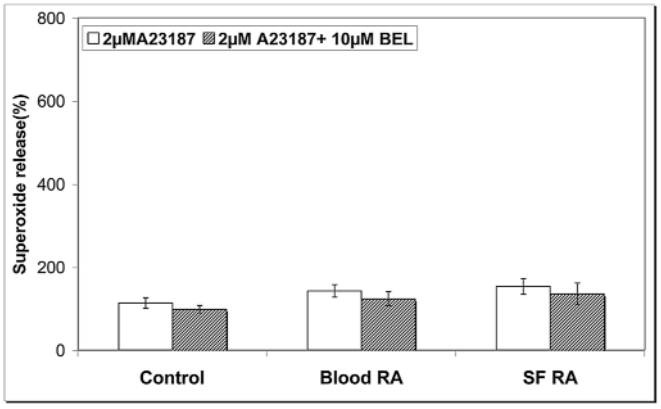
trophils stimulated with either A23187 or PMA is shown in Fig. 4B. The superoxide anion production by peripheral RA-PMNs stimulated with PMA was inhibited in a concentration-dependent manner by BEL; significant inhibitions were obtained when 0.1 up to 10μM of BEL were used. In contrast, the effect of 2μM A23187 was not significantly altered by BEL treatment at concentrations up to 10μM (Fig. 4B).

To test which PLA_2 isoform is involved in the mechanism of PMA or A23187-induced stimulation of neutrophils, the production of superoxide anion by stimulated cells was measured in the presence or absence of AACOCF3 or BEL.

Effect of Ca^{2+}-dependent inhibitor AACOCF3 on superoxide anion production by RA-PMNs

20μM AACOCF3 inhibited superoxide anion production by PMNs isolated from peripheral blood or SF of RA patients, activated with calcium ionophore (2μM A23187), as shown in Fig. 5A whereas PMA-dependent superoxide anion release was not affected (Fig. 5B). Consistent with these results was the finding that PLA_2 mediated superoxide anion production was specifically stimulated by PMA, but no significant release of superoxide anion was seen in PMNs-RA after A23187 treatment, and that calcium ionophore effect was decreased by 20μM AACOCF3 treatment.

Synovial and peripheral blood PMNs isolated from RA patients show a slight increase in superoxide anion production when activated with A23187, but they respond to AACOCF3 (Fig. 5A) more intensly than peripheral normal blood PMNs do ($p<0.05$). These results suggest that the effect A23187 has on the oxidative activity of RA-PMNs might be up-regulated by Ca^{+2} -dependent PLA_2 activation, whereas effect of PMA on the superoxide anion release is not mediated by this PLA_2 isoform (Fig. 5B).



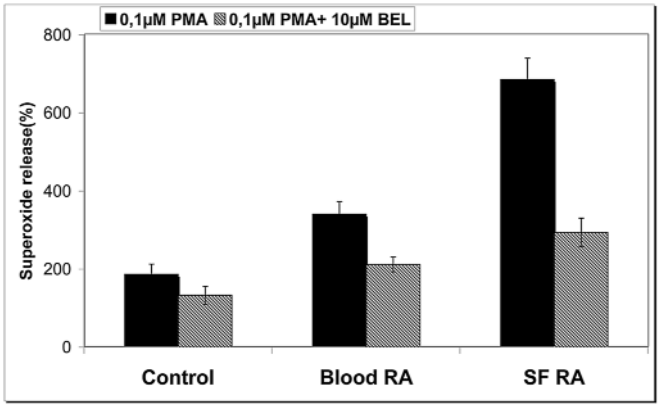


Fig. 6. Inhibition of calcium ionophore or PMA activity on superoxide anion release by AACOCF3 or BEL. Peripheral blood PMNs from RA-patients, were incubated for 15 min in the presence or absence of scalar concentrations of AACOCF3 (panel A) or BEL (panel B), and stimulated for 20 min with 2µM A23187 or 0.1µM PMA. The cellular response developed by RA-PMNs in the absence of stimuli was considered to be 100%, n=10 in each group.

Effect of BEL on superoxide anion production by RA-PMNs

The superoxide anion production induced by 2µM A23187 was not significantly altered by BEL treatment, neither in peripheral blood nor in synovial fluid PMNs (Fig. 6A). In contrast, 10µM BEL inhibit superoxide anion release by peripheral and synovial PMNs activated with PMA (Fig. 6B). Results show a significant difference between PMNs isolated from normal blood and RA-PMNs, isolated either from blood ($p<0.005$) or from synovial fluid ($p<0.001$), in PMA stimulated superoxide anion production.

In light of the reported specificity of BEL for calcium-independent PLA_2 activity, the inhibition of PMA-induced superoxide anion by BEL (Fig. 6B) is consistent with the hypothesis that oxidative response to PMA is due to activation of a calcium-independent isoform of PLA_2.

Effects of arachidonic acid on the superoxide anion production by RA-PMNs

Since AACOCF3 and BEL could inhibit PLA_2 activity and modulate the superoxide anion production by RA PMNs, we have investigated whether metabolites such as AA, which results from PLA_2 activity, would overcome the inhibitory effect induced by AACOCF3 or BEL. Addition of 0.1 to 100µM exogenous AA did not seem to amplify the superoxide anion release by RA-PMNs, previously stimulated with A23187, as shown in Fig. 7. Unexpectedly, the AA amplifies the effect PMA has on superoxide anion release by peripheral RA-PMNs with a maximal response at a concentration of 10µM AA ($p<0.0005$) (Fig.7).

To confirm that AA interferes with the inhibitory effect of AACOCF3 or BEL on the oxidative activity developed by RA PMNs, we examined the effects induced by AA treatment on the superoxide anion release by A23187 or PMA activated neutrophils, in

the presence or absence of the above inhibitors. As shown in Fig. 8A, the inhibitory effect of AACOCF3 on PLA_2 activity and superoxide anion release by RA-PMNs could be partially restored by the addition of exogenous AA. Synovial and peripheral blood RA-PMNs, activated by A23187 in the presence of AACOCF3, respond to AA in the same manner as normal peripheral PMNs. On the other hand, AA amplifies PMA effects on superoxide anion release by RA-PMNs to a greater extent than it does for A23187, and could overcome the inhibitory effect of BEL (Fig. 8 A, B).

The effect of AA is significantly stronger for synovial fluid RA neutrophils, as compared to peripheral blood RA-PMNs ($p<0.001$), both types of cells being previously treated with PMA and BEL (Fig. 8B). Results show a statistical difference between levels of superoxide anion release by PMNs isolated from controls and RA-PMNs isolated from blood ($p<0.005$) or SF ($p<0.005$), co-stimulated with PMA and BEL, in the presence of AA (Fig. 8B). This phenomenon involves AA, which is generated by PLA_2 activation during the superoxide anion production by RA PMNs. Based on these observations, we suggest that AA might be a participant in the PLA_2-dependent regulation of NADPH oxidase activity, and modulates the superoxide anion production.

Discussion

PMNs recruited in the synovial cavity of RA patients are activated and have the capacity to amplify damage that joints sustain by releasing of proteases and oxygen/nitrogen metabolites [9]. Our experimental data indicate that synovial RA PMNs develop a more intense respiratory burst as compared to normal cells.

In the present study both PMA and calcium ionophore A23187 stimulated the release of superoxide anion by neutrophils isolated from patients with rheumatoid arthritis; however, the characteristics of superoxide anion release in response to those two agents were markedly different. Moreover, the oxidative response of neutrophils to specific inhibitors was different, suggesting that different isoforms of PLA_2 were activated by A23187 or PMA, and that activation of these PLA_2s isoforms serves distinct functions within the RA-PMNs. In rheumatoid arthritis, A23187 has reduced stimulatory activity on superoxide anion release through a calcium-dependent mechanism that could be inhibited by ACCOCF3. In contrast, PMA has strongly stimulated oxidative response through a calcium – independent mechanism, inhibited by BEL addition. AA amplified the effect PMA has on synovial RA PMNs superoxide anion release, but did not modify the cellular response to A23187. Taken together, these data suggest a possible correlation between the superoxide anion production by A23187 or PMA - stimulated RA-PMNs, and the metabolic pathways responsible for AA production.

Our results suggest that RA is associated with functional changes of PMNs as regards the oxidative response to PMA challenge. The susceptibility of RA patients to infections might be due to the impairment of PMA triggered oxidative burst. However, the increase in intracellular calcium induced by A23187, was not enough to induce superoxide anion release, suggesting that other cellular changes contribute to the toxic response, such as membrane lipid peroxidation together with PLA_2 activation.

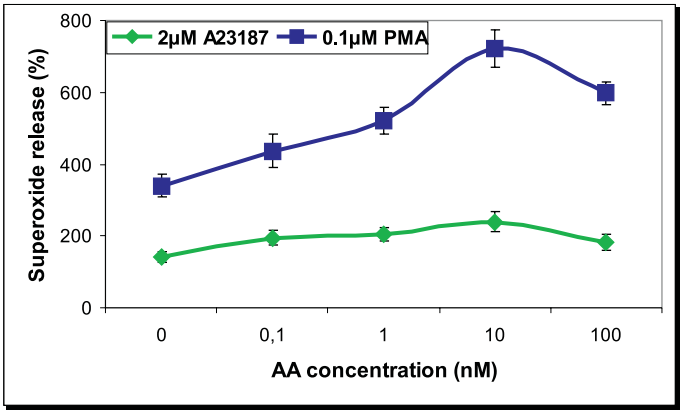


Fig. 7. Effect of exogenous AA on superoxide anion release by peripheral blood PMNs from RA patients, activated *in vitro* with 2μM A23187 or 0.1μM PMA. The cellular response developed by RA PMNs in the absence of stimuli was considered to be 100%, n=10 in each group.

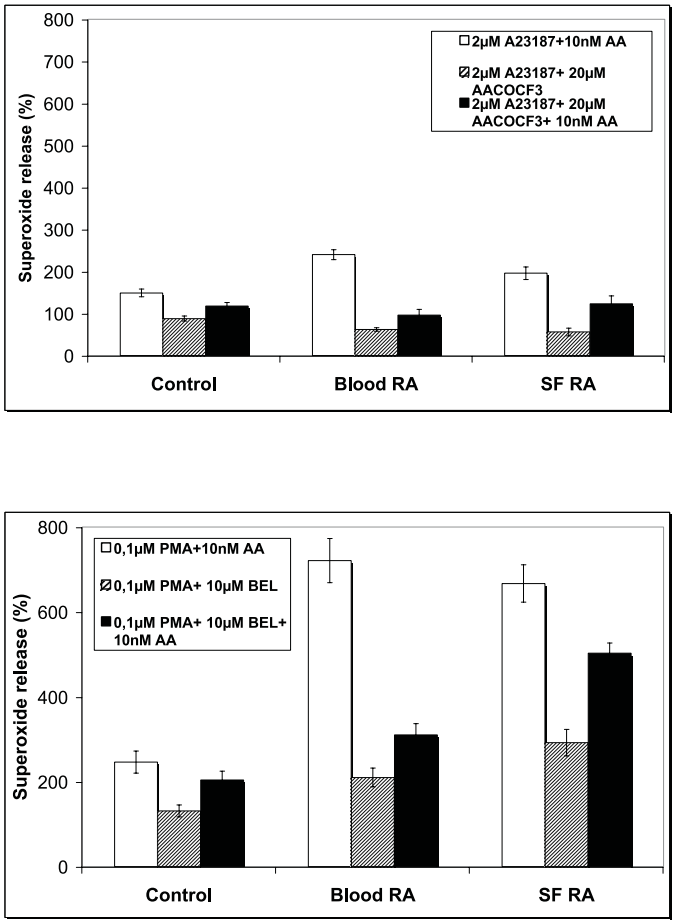


Fig. 8. Exogenous AA reverses AACOCF3- and BEL-induced inhibition of superoxide anion release by peripheral and SF PMNs from RA patients, activated *in vitro* with 2μM A23187 (panel A) or 0.1μM PMA (panel B), as compared to healthy donors. The cellular response developed by PMNs in the absence of stimuli was considered to be 100%, n=10 in each group.

The effect of Ca -dependent (ACCOCF3) or Ca-independent (BEL) specific PLA_2 inhibitors, on the superoxide anion production are consistent with the hypothesis that in RA neutrophils activation of calcium-dependent PLA_2 does not lead to amplification of oxidative response, while activation of calcium-independent PLA_2 contributes to increase the oxidative activity in the swallen joint.

In conclusion, the respiratory burst of PMNs isolated from RA patients might trigger significant oxygen-dependent microbicidal activity and oxidative stress both in periphery and in the inflammatory joint. Our results demonstrate that PLA_2 may be involved in the regulation of NADPH activity in RA-PMNs and plays a central role in PMA-stimulated superoxide anion production, particulary in the inflammatory situs. Our data will perhaps lead to a better understanding of pathological phenomena, that characterize RA.

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