Regulation of the arachidonic acid-stimulated respiratory burst in neutrophils by intracellular and extracellular calcium

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Abstract **The respiratory burst is an important physiological function of the neutrophils in killing the bacteria invading in human body. We used chemiluminescence method to measure the exogenous arachidonic acid-stimulated respiratory burst, and measured the cytosolic free calcium concentration in neutrophils by the fluorescence method. It was found that, on one hand, the arachidonic acid-stimulated respiratory burst was enhanced by elevating the cytosolic free calcium concentration in neutrophils with a potent endomembrane Ca^{2+}-ATPase inhibitor, Thapsgargin; on the other hand, chelating the intracellular or extracellular calcium by EGTA or BAPTA inhibited the respiratory burst. Results showed that calcium plays an important regulatory role in the signaling pathway involved in the exogenous arachidonic acid-stimulated respiratory burst of neutrophils.**

Keywords: **arachidonic acid, neutrophils, respiratory burst, chemiluminescence, intracellular calcium, extracellular calcium.**

Neutrophils are important leukocytes in the defense system of human body. They kill the invaded bacteria by phagocytosis and release of large amount of superoxide anions (O_2^-) and its derived oxygen radicals. In this process, neutrophil consumes huge amount of oxygen via hexose monophosphate shunt. Thus, the phenomenon is often called "respiratory burst"[1]. Arachidonic acid exists in neutrophils as one of the important fatty acids. The endogenous arachidonic acid is the precursor to a plethora of eicosanoids and other bioactive molecules. The exogenous arachidonic acid can stimulate neutrophils to undergo respiratory burst[2, 3]. In the past years, increasingly accumulated experimental evidence showed that arachidonic acid played an important pathological role in the myocardial cell damage caused by ischaemia-reperfusion. It was reported in 1984 that ischaemia of myocardium leads to degradation of the phospholipids on cell membrane and the accumulation of fatty acids including arachidonic acid, and reperfusion of the ischemic myocardium results in its further accumulation[4–6]. It is also found that for some reason neutrophils accumulate in the ischemic myocardium. In the ischemic tissue, neutrophils are activated by the accumulated arachidonic acid and some complement fragments undergo respiratory burst and release considerable O_2^- and oxygen radicals. It is those radicals that interfere the contraction of myocardial cells, damage mitochondria and plasma membrane, and result in an irreversible injury of the myocardial cells[7, 8]. Therefore, to study the signal transduction in arachidonic acid-stimulated respiratory burst would be certainly necessary for understanding the mechanisms involved in the ischaemis-reperfusion injury of myocardium and seeking protection measure to minimize the injury.

It used to be believed that arachidonic acid stimulates respiratory burst in neutrophils by directly activating protein kinase C (PKC), and that the AA-stimulated respiratory burst is cacium-independent[9]. In the last few years, there were some reports that arachdonic acid can cause cytoplasmic calcium mobilization[10] and exogenous nucleotides, such as ATP, can induce an elevation in intracellular Ca^{2+} and enhance the respiratory burst stimulated by fMLP in neutrophils[11]. However, no systematic investigation on regulatory role of intracellular and extracellular calcium in arachidonic acid-stimulated respiratory burst of neutrophil has been reported. In the present investigation, we studied the regulation of the arachidonic acid-stimulated respiratory burst by intra- and extracellular calcium. In the studies, chemiluminescence method was used to measure the respiratory burst of neutrophils and its kinetics, the intracellular Ca^{2+} concentration in cells was measured by utilizing the fluorescent probe fura-2. Thapsigargin, an inhibitor of endomembrane Ca^{2+}-ATPase, was used to elevate the intracellular Ca^{2+}. BAPTA-AM, the membrane permeable chelator for intracellular calcium, and EGTA, the chelator of extracellular calcium, were used to chelate the intracellular or extracellular Ca^{2+} respectively. Panax notoginseng saponins (PNGS), a blocker of Ca^{2+}-channel on the plasma membrane, was used to block the Ca^{2+} entry through channels. The present study provided some new insight into the signal pathways in arachidonic acid-stimulated respiratory burst in human neutrophils.

1 Materials and methods

(i) Reagents. Arachidonic acid, Thapsigargin, EGTA were all purchased from Sigma. Fura-2/AM, BAPTA/AM were from Molecular Probe. Panax notoginseng saponins (PNGS) were purchased from Yunnan Phytopharmaceutical (Kunming, China). It mainly consists of panaxadiol and panaxatriol. Other reagents are all of analytically grade. The Hanks' balanced salt solution (HBSS) consists 8 g/L NaCl, 0.4 g/L KCl, 1 g/L glucose, 60 mg/L KH_2PO_4, 47.5 mg/L Na_2HPO_4, pH=7.2.

(ii) Isolation of neutrophils. Neutrophils were isolated from fresh blood of healthy donor. The detailed

procedure of the cell preparation can be found in ref. [12].

(iii) Measurement of the respiratory burst. The respiratory burst of neutrophils was measured by chemiluminescence. Cells were suspended in the HBSS with a concentration of 1×10^6 cells/mL. Luminol (100 ng / mL) was added to the cell suspension before measurement. Each of 1 mL cell suspension was put into two identical quartz cuvettes, one was used as testing sample, and the other as control. They were placed in a rotatable sample holder of a laboratory-made photon counter and measured at 37℃. When the cells underwent respiratory burst, the superoxide and H_2O_2 generated in the cell suspension reacted with luminol to emit photons, which were received by a photon multiplier. Since respiratory burst of the cells for study and the respiratory burst of cells as control were measured simultaneously, the errors induced by the time-dependent variation in cell vitality, kinetics, and intensity of respiratory burst were minimized to a great extent.

(iv) Measurement of the cytosolic free calcium concentration. According to the method in ref. [13], neutrophils (10^6 cells/mL) were loaded with fura-2 by incubating cells with 1 μmol/L fura-2/AM at 37℃ for 45 min. Thereafter, the cells were washed twice to remove the extracellular dye and resuspended in HBSS at a density of 10^6 cells/mL. The loaded neutrophils were added in magnetic stirring cuvette and measured at 37℃ on a dual excitation fluorescence spectrophotometer (Hitachi F-4500) which allowed simultaneous excitation of fluorescence at 340 and 380 nm. During the measurement, TG was injected into the cell suspension. The fluorescence of the cytoplasmic Ca^{2+}-binding fura-2 excited at 340 and 380 nm, F_{340} and F_{380}, were simultaneously recorded at the emission of 510 nm. The free cytoplasmic calcium concentration, $[Ca^{2+}]_i$, in cells after the treatment with TG was calculated according to the following equation:

$$[Ca^{2+}]_i = K_d\,[(R-R_{min})/(R_{max}-R)]\,(F_{380,min}/F_{380,max}),$$

where K_d is the dissociation constant for Ca^{2+} of fura-2(224 nmol/L); $R=F_{340}/F_{380}$; $R_{max}=F_{340,max}/F_{380,max}$; $R_{min}=F_{340,min}/F_{380,min}$; $F_{340,max}$ and $F_{380,max}$ are the fluorescence excited at 340 and 380 nm, respectively, at saturated Ca^{2+} concentration achieved by addition of 2 μL Triton in 2 mL cell suspension. $F_{340,min}$ and $F_{380,min}$ are the fluorescence excited at 340 and 380 nm, respectively, at zero Ca^{2+} concentration achieved by addition of 2 mmol/L EGTA in cell suspension.

2 Results and discussion

(i) Effect of the steady-state cytosolic Ca^{2+} concentration on the AA-stimulated respiratory burst in neutrophils. We recorded the concentration of cytoplasmic free Ca^{2+} after adding different concentrations of TG to the cell suspension. As shown in fig. 1(b), after addition of TG, the $[Ca^{2+}]_i$ increased gradually and reached stable in about 600 s. When the concentration of TG increased, the elevation of the $[Ca^{2+}]_i$ was greater. The $[Ca^{2+}]_i$ was elevated from 120 to 600 nmol/L about 10 min after adding 500 nmol/L TG to the cell suspension. Accordingly, the corresponding change of the AA-stimulated respiratory burst of neutrophils pretreated with a different concentration of TG was recorded. As shown in fig. 2(a), it can be seen clearly that, as the steady-state calcium concentration was elevated, the corresponding AA-stimulated respiratory burst was enhanced. The intensity of the AA-induced respiratory burst of neutrophils pretreated with 500 nmol/L TG for 10 min was almost five times as much as the control (without the pretreatment of TG and stimulated with the same concentration of AA). The higher the concentration of TG, the more significantly the $[Ca^{2+}]_i$ was elevated, and consequently the more intensive the respiratory burst was. However, no change in the kinetic of respiratory burst was found as $[Ca^{2+}]_i$ increased. The experimental result showed that the signaling pathways involved in the AA-induced respiratory burst of neutrophils might be calcium-dependent.

(ii) Effect of chelator of intracellular and extracellular calcium on the AA-induced respiratory burst of neutrophils. To further investigate the dependence of the AA-stimulated respiratory burst on the intra- and extracellular calcium, we recorded the AA-induced respiratory burst 5 min after depleting the intracellular calcium or chelating the extracellular calcium with BAPTA and EGTA respectively. As shown in fig. 2, pretreatment of the cells with 2 mmol/L EGTA or 5 μmol/L BAPTA resulted in about 80% inhibition of the AA-induced respiratory burst. Depleting the intracellular Ca^{2+} or chelating the extracellular Ca^{2+} alone cannot affect the kinetic of the respiratory burst. However, when 2 mmol/L EGTA and 5 μmol/L BAPTA were both present in the cell suspension, the AA-induced respiratory burst was completely inhibited. At one aspect, the results showed that the AA-stimulated respiratory burst was calcium-dependent. Cells could no longer undergo respiratory burst when the intracellular and extracellular free calcium were both chelated. At the other aspect, the results indicated that the full activation needed the presence of both intra- and extracellular calcium, the absence of either would inhibit largely but not completely the respiratory burst.

(iii) Inhibition of the AA-induced respiratory burst in neutrophils by PNGS, a blocker of Ca^{2+}-channel. To further study the regulatory role played by the extracellular calcium in the AA-stimulated respiratory burst, the AA-induced respiratory burst after pretreating neutrophils with different concentrations of PNGS was measured (fig. 3). The main composition in PNGS, panaxatriol and

panaxadiol was all triterpenoid. They are blockers of the Ca^{2+}-channel on the cell membrane and can inhibit the influx of the extracellular calcium into cells[14]. As shown in fig. 3, the AA-stimulated respiratory burst was inhibited by 50% after the treatment of neutrophils with 100 μg/mL PNGS for 5 min. 200 and 500 μg/mL PNGS

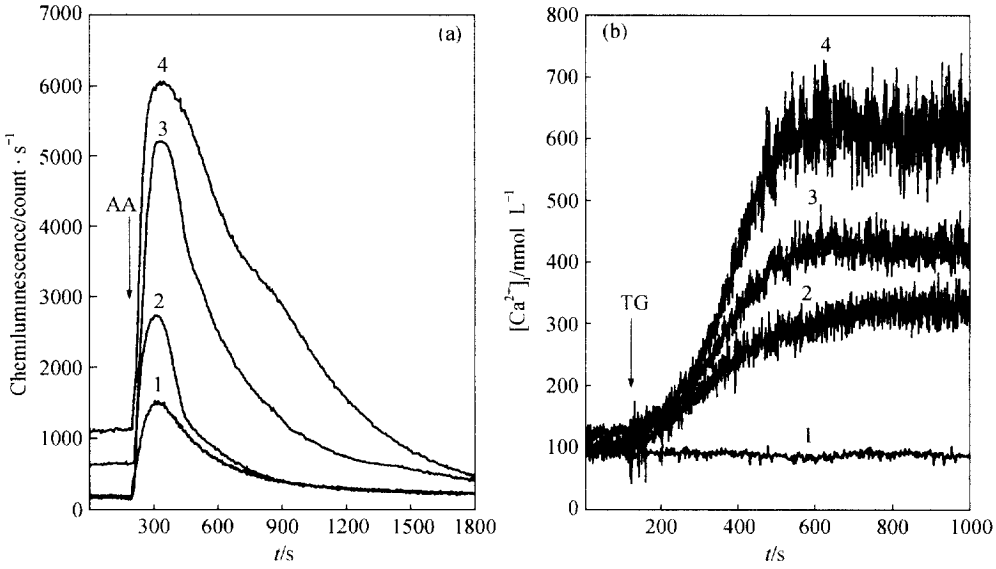

Fig.1. The corresponding 50 μmol/L AA-stimulated respiratory burst (a) and the intracellular free calcium concentration (b) in neutrophils treated with no TG (1), 20 nmol/L (2), 100 nmol/L (3), and 500 nmol/L TG (4). Neutrophils (10^6 cells/mL) were suspended in HBSS containing 1.3 mmol/L Ca^{2+} Measurement was carried out at 37℃ The burst was measured as concomitant chemiluminescence.

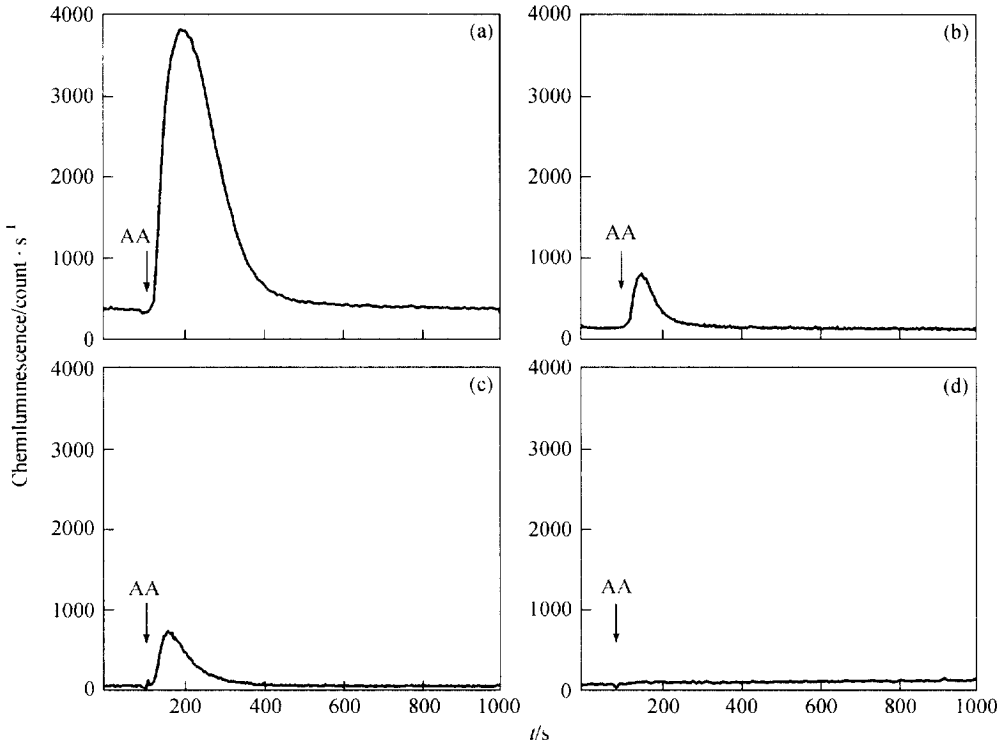

Fig. 2. The AA-stimulated respiratory burst in the neutrophils in the absence (a) or presence of 2 mmol/L EGTA (b), 5 μmol/L BAPTA (c), 2 mmol/L EGTA and 5 μmol/L BAPTA (d). 50 μmol/L AA was added to the cell suspension (in HBSS) 5 min after the incubation of cells with EGTA or BAPTA The burst was measured as concomitant chemiluminescence at 37℃.

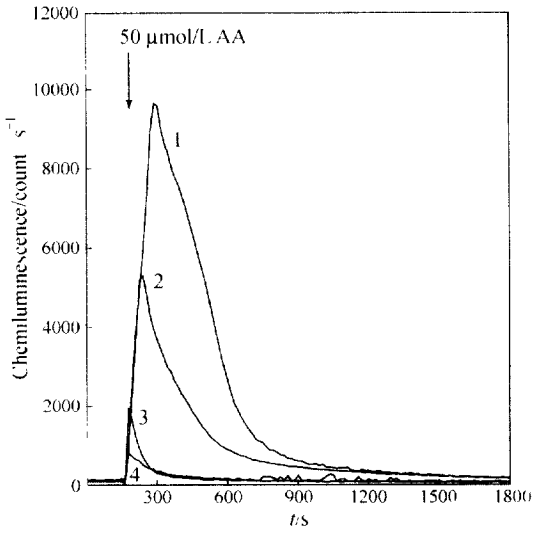

Fig. 3. The AA-induced respiratory burst in neutrophils pretreated with no PNGS (1), 100 μg/mL (2), 200 μg/mL (3), and 500 μg/mL PNGS (4). 50 μmol/L AA was added to the cell suspension (in HBSS) 5 min after the treatment of cells with the PNGS. The burst was measured as concomitant chemiluminescence at 37 °C.

can inhibit the respiratory burst by 80% and 92% respectively. However, when the concentration of the PNGS exceeded 500 μg/mL, no more inhibition was found (data not shown). This result shows that when all the calcium channels on the cell membrane were blocked by PNGS, namely the calcium influx was completely inhibited, the resting respiratory burst was maintained only by the calcium released from the intracellular calcium store. The inhibition of the respiratory burst by PNGS indicates the importance of the calcium influx from extracelluar space in the signaling pathway of the activation of neutrophils. AA can induce calcium influx in many cells including neutrophils, and consequently leads to a great increase in cytosolic calcium concentration, which was already reported[15, 16]. However, the relationship between the calcium influx and the respiratory burst has not been studied. Our experimental results showed that, the AA-stimulated respiratory burst needs the cooperation of the intra- and extracellular calcium, and such a cooperation is achieved through the influx of the extracellular calcium.

3 Conclusion

In the AA-stimulated respiratory burst of neutrophils, intracellular and extracellular calcium plays an important regulatory role. On one hand, the increase of the cytosolic calcium concentration significantly enhances respiratory burst; on the other hand, the calcium influx plays a very important role in maintaining the transient increase of the cytosolic calcium concentration and the stimulation of the respiratory burst. Thus, it can be concluded that the AA-induced respiratory burst is very much dependent on the intracellular and extracellular calcium.

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