

The acute phase protein serum amyloid A primes neutrophils

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Received 20 January 2003; received in revised form 21 March 2003; accepted 25 March 2003

First published online 18 April 2003

Abstract

We studied here the effect of the acute phase protein serum amyloid A (SAA) on the oxidative burst of neutrophils. Incubation of neutrophils with SAA increased the rate of oxygen uptake and the production of reactive oxygen species of neutrophils activated with opsonized zymosan (OZ). The increment in the neutrophil oxidative burst was dependent on SAA concentration in the range of 3–33 $\mu\text{g protein ml}^{-1}$ and was observed only in the presence of a relatively low amount of OZ (1×10^6 particles ml^{-1}). SAA did not affect oxygen consumption and reactive oxygen production triggered by other stimuli, such as f-Met-Leu-Phe, phorbol myristate acetate or non-opsonized zymosan. Our finding points to a priming effect of SAA probably associated with mobilization of receptors for opsonized particles and strengthens the role of SAA as an effector of neutrophil functions in inflammation.

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Keywords: Serum amyloid A; Neutrophil; Acute phase protein; Oxidative burst; Luminol-enhanced chemiluminescence; Priming; Reactive oxygen species

1. Introduction

Serum amyloid A (SAA) is an acute phase protein synthesized by human liver in response to various systemic injuries [1]. In inflammation, the serum concentration of SAA may reach levels up to 1000-fold greater than that found in the non-inflammatory state [2]. Although the SAA protein is very well conserved throughout evolution and its increase in inflammation is so expressive, the biological role of SAA has only begun to be elucidated [3–5].

While the increase in the serum level of SAA is the systemic side of the inflammation, the migration of leukocytes to the inflamed tissue is one of the early events of the localized response. Neutrophils are the first cells that migrate into tissues in response to invading pathogens. The antimicrobial function of these phagocytes depends on the release of lytic enzymes stored in cytoplasmic granules and on the generation of superoxide anion and other derived highly reactive oxidants. Superoxide anion is generated at

the expenses of oxygen and NADPH by the phagocyte NADPH oxidase system during a process designated oxidative burst [6,7].

The temporal coincidence between neutrophil migration and the hepatic synthesis and release of SAA supported the investigation of the effects exerted by SAA on neutrophil functions. We previously studied the effect of SAA on the release of tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-8 by human neutrophils and observed that SAA is a potent stimulus for the release of these three cytokines [8]. In this study we verified the effect of SAA on the neutrophil oxidative burst.

2. Materials and methods

2.1. Reagents

Dextran, Histopaque[®], lipopolysaccharide (*Escherichia coli* 026:B6), and luminol were from Sigma (St. Louis, MO, USA). Recombinant human SAA was from Peprotech (Rocky Hill, NJ, USA), human anti-TNF- α from Calbiochem (San Diego, CA, USA) and platelet-activating factor (PAF) from Cayman (Ann Arbor, MI, USA). All other chemicals and reagents were of reagent grade.

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2.2. Cell purification and culture

Neutrophils (>99% purity) were isolated from the peripheral blood of healthy donors under endotoxin-free conditions as used elsewhere [8]. After purification, leukocytes were suspended in phosphate-buffered saline (PBS) pH 7.4 enriched with 1 mg ml⁻¹ glucose, 1 mM CaCl₂ and 0.5 mM MgCl₂, counted in a Neubauer chamber and immediately used.

2.3. Oxygen consumption

Neutrophils (2.0 × 10⁶ ml⁻¹) were suspended in PBS and incubated in a YSI-53000 Biological Oxygen Monitor chamber, final volume 3 ml, at 37°C with or without SAA (3–33 µg ml⁻¹) for 5 min. Subsequently, f-Met-Leu-Phe (fMLP, 0.1 and 1 µM), opsonized or non-opsonized zymosan (1.0 × 10⁶–1.0 × 10⁷ particles ml⁻¹) was added as stimulus. The results are given as maximal rates of oxygen consumption obtained after the addition of the stimulus as previously described [12]. A positive control was run at the same conditions using 0.1 µM PAF as the priming agent.

2.4. Oxidative burst

The effect of SAA (10 µg ml⁻¹) in the neutrophil oxidative burst was evaluated by the luminol chemiluminescence assay. Neutrophils (2.5 × 10⁶ ml⁻¹) pre-incubated with SAA for 5 min were stimulated with zymosan (1 × 10⁶–1 × 10⁷ particles ml⁻¹) or phorbol myristate acetate (PMA, 1.6–16 ng ml⁻¹) in the presence of luminol (1 mM). The reaction was run in PBS buffer at 37°C in a final volume of 0.3 ml. Chemiluminescence was followed in an EG&G Berthold LB96V microplate luminometer.

2.5. Statistical analysis

Comparisons were done by ANOVA, with the Student–Newman–Keuls multiple comparisons test.

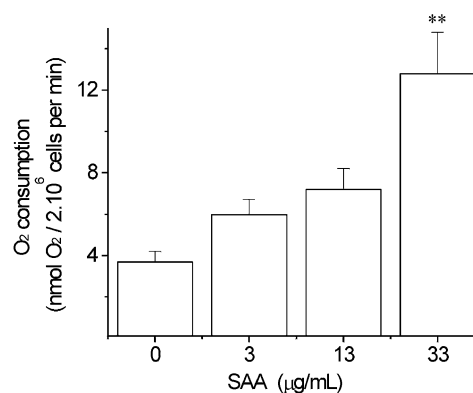


Fig. 1. SAA increases the rate of oxygen consumption of neutrophils (2 × 10⁶ cells ml⁻¹) activated by OZ (1 × 10⁶ particles ml⁻¹). The data represent the average ± S.D. of the maximal rates of oxygen consumption obtained after the addition of the stimulus in three to five experiments. The reaction was carried out in PBS pH 7.4 at 37°C. The final volume was 3 ml.

3. Results and discussion

SAA by itself did not trigger oxygen consumption in neutrophils (Table 1). On the other hand, it affected the rate of oxygen consumption triggered by opsonized zymosan (OZ) (Table 1 and Fig. 1). A positive control with the same conditions used for SAA was run with PAF (0.1 µM) as a priming agent and OZ as the stimulus. In the presence of PAF, neutrophils consumed 13.0 ± 3.1 nmol O₂ per 2 × 10⁶ cells min⁻¹ (average ± S.D. of three experiments), which is 3.5 times higher than the oxygen consumed without PAF. The increase of the neutrophil response to OZ caused by SAA agrees with the definition of priming neutrophils. Priming is a state of preactivation of dormant neutrophils that enables a more powerful response to be generated once microbicidal activity is initiated [7]. Classical priming agents such as cytokines and eicosanoids [9–12] are not able by themselves to stimulate neutrophil oxygen consumption but cause an increment in the maximal rate of oxygen consumption when a second stimulus is added. The amplification of the neutrophil oxidative burst

Table 1
Effect of SAA on the oxygen consumed by stimulated neutrophils

Stimulus	Oxygen consumption nmol O ₂ /2 × 10 ⁶ cells per min	
	SAA (µg ml ⁻¹)	
	-	33 µg ml ⁻¹
None	nd	nd
Opsonized zymosan (1 × 10 ⁶ particles ml ⁻¹)	3.7 ± 0.5	10.8 ± 2.5**
Non-opsonized zymosan (1 × 10 ⁶ particles ml ⁻¹)	1.5 ± 1.0	3.0 ± 1.0
fMLP (1 µg ml ⁻¹)	5.3 ± 0.7	5.2 ± 0.3
fMLP (0.1 µg ml ⁻¹)	nd	nd

Data are the average ± S.D. of the amount of oxygen consumed (nmol/2 × 10⁶ cells min⁻¹) of five experiments. The reactions were carried out with 2 × 10⁶ neutrophils ml⁻¹ in PBS pH 7.4, 37°C, final volume 3 ml. When added, SAA was pre-incubated with cells for 5 min prior to the addition of the stimulus.

nd = not detectable.

**P ≤ 0.005 compared with the absence of SAA.

in response to OZ by previous exposure of the cell to SAA defines SAA as a priming agent.

Given that it is known that SAA induces the release of cytokines by neutrophils [8] and some of these cytokines prime neutrophils [13], an indirect effect of SAA should be expected at longer times of incubation with neutrophils. To preclude this indirect effect, SAA was incubated with neutrophils for a short time (5 min). Furthermore, we excluded the participation of TNF- α (one of the former cytokines released by neutrophils) because the addition of anti-TNF- α ($10 \mu\text{g ml}^{-1}$) to the assay did not preclude the priming effect of SAA. The effect of SAA as a priming agent was dose-dependent in the range of 3–33 $\mu\text{g protein ml}^{-1}$ (Fig. 1). The range of SAA assayed was much lower than that present in serum in the acute phase response ($100\text{--}2000 \mu\text{g ml}^{-1}$) [14], indicating that in vivo conditions may afford enough SAA to prime neutrophils.

The priming effect of SAA seemed to be exclusive to steps involved in the recognition of opsonized particles because the incubation of neutrophils with SAA did not affect the consumption of oxygen triggered by other types of stimuli, such as non-opsonized zymosan or fMLP (0.1 and $1 \mu\text{M}$) (Table 1). Further evidence for the role of SAA in the process of recognition of opsonized particles was found in the increased production of reactive oxygen species during the oxidative burst triggered by OZ and measured by the luminol-enhanced chemiluminescence (Fig. 2A). It should be noted that the incremental effect of

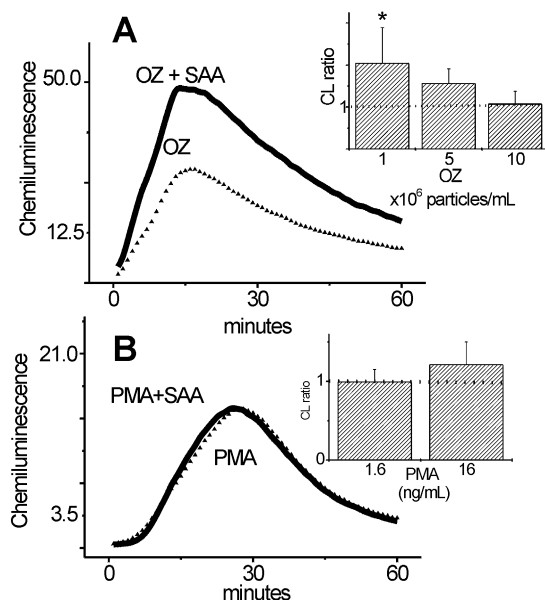


Fig. 2. Light emission kinetics from neutrophils (2×10^6 cells ml^{-1}) stimulated with 1×10^6 particles of OZ ml^{-1} (A) or 16 ng ml^{-1} of PMA (B) in the presence of luminol (1 mM). Pretreatment of the neutrophils with SAA ($33 \mu\text{g ml}^{-1}$) causes an increase in the chemiluminescence of neutrophils stimulated with OZ but not with PMA. The insets represent the average \pm S.D. of the ratio between the integrated light emission in the presence and absence of SAA at different amounts of the respective stimulus of five experiments. * $P \leq 0.01$ in comparison with the experiments without SAA.

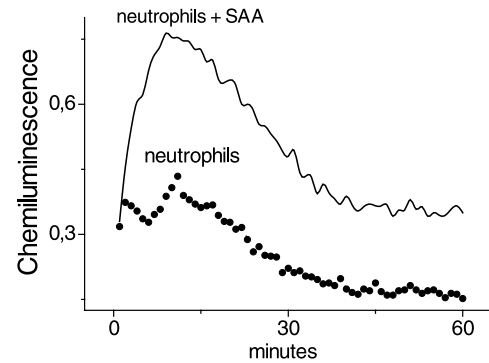


Fig. 3. Light emission kinetics from non-stimulated neutrophils (2×10^6 cells ml^{-1}) in the presence of luminol (1 mM). SAA caused a slight increase in chemiluminescence. The inset represents the average \pm S.D. of the ratio between integrated light emission in the presence and absence of SAA of five experiments. $P \leq 0.001$.

SAA was only observable at low concentrations of the stimulus; the effect of SAA is evident when 1×10^6 particles of OZ were added as stimulus but not for higher amounts of OZ (Fig. 2A, inset). This same pattern of response is commonly observed with other priming agents. Usually the priming effect becomes apparent only when the cells are subsequently stimulated with low concentrations of the stimulus. The apparent specificity of SAA as a priming agent was strengthened by the absence of effect of SAA on the production of reactive oxygen species triggered by non-opsonized zymosan (data not shown) or PMA (1.6 and 16 ng ml^{-1}) (Fig. 2B).

Finally, it was also observed that although SAA was unable to trigger a chemiluminescent signal of the magnitude expected for classical stimuli (around three or four orders of magnitude higher than non-stimulated cells), it caused a slight increase (two to three times) in the basal chemiluminescence (compare scales of Fig. 3 with Fig. 2). Although this is a side point in this study, the increase in the chemiluminescence in the presence of SAA is a reproducible finding and is consistent with the previous description of a weak but detectable increase in the neutrophil production of superoxide anion in the presence of SAA using a fluorescent probe [15]. The origin and biological significance of the slight increase in the intracellular pool of reactive oxygen species produced by SAA in neutrophils has not been elucidated yet.

In serum, SAA associates with the high-density lipoprotein fraction [3], a relatively small particle ($75\text{--}120 \text{ \AA}$) that may have easier access to the inflammatory site than other lipoproteins. A localized source of SAA might also be provided by monocytes/macrophages [16] present in the inflammatory focus. That SAA is present in the inflammatory site is shown by the presence of SAA-derived peptides in human rheumatic synovial fluids [17]. Although the primary physiologic role of SAA remains obscure there is a set of studies that indicate the action of SAA on the modulation of leukocyte functions. In neutrophils, SAA induces chemotaxis and adhesion to endothelial cells

[18], triggers a rapid and transient increase of cytosolic calcium, up-regulates the cell surface expression of CD11 and CD16, increases secretion of lactoferrin [15], stimulates the release of proinflammatory cytokines and IL-8 [8] and directly primes neutrophils to opsonized particles (this study). From the entire set of studies with SAA and neutrophils, it seems that the induction of activation processes prevails.

SAA and C reactive protein (CRP) are the main human acute phase proteins. The biological function of CRP appears to be host defence against bacterial pathogens. CRP interacts with Fc receptors on phagocytic cells and acts like an opsonin [19]. Although the activities described for SAA are different from that reported for CRP, the finding that SAA primes neutrophils, especially in response to opsonized particles, suggests that these two acute phase proteins have a concerted mode of action driving a more powerful response of innate host defense.

Acknowledgements

The authors are indebted to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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