Australian Journal of Basic and Applied Sciences, 6(6): 246-250, 2012
ISSN 1991-8178

Regulation of Arachidonic Acid Release by Some Agonists on Suspended and Attached Neutrophils

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Abstract: [^{3}H] Arachidonic acid is released after stimulation of suspended and attached neutrophils. The arachidonic acid release in attached cells is more than in suspended cells. This release is rapid with the increase of incubation time and dose depended. The Fmet-Leu-Phe (FMLP) potentiates [^{3}H] arachidonic acid release in attached cells pretreated with LPS and serum. [^{3}H] arachidonic acid release was less in suspended cells in comparison to attached cells where the addition of low concentration of LPS in presence of serum or platelet activating factor (PAF) to suspended cells produce increase in arachidonic acid release, where lps in combination with PAF produce a large and significant increase in arachidonic acid release. The potentiative effect of LPS is mediated via CD14 receptors. The monoclonal antibodies against CD14 had no effect on arachidonic acid release in PAF treated cells where it inhibited greatly the potentiation by LPS or LPS-serum complex with PAF.

Key words:

INTRODUCTION

The neutrophils release arachidonic acid (AA) in response to stimulation. The fatty acid is then metabolized to generate one or more biologically active eicosanoids such as prostaglandins, prostacycline, thromboxanes and leukotrienes (Astudillo *et al*., 2012).

Platelet activating factor (PAF) possesses multiple biological activities which encompasses the field of allergic and inflammatory reactions as well as the regulation of the cardiovascular system. The effect of PAF on neutrophils includes chemotaxis, degranulaion, aggregation, oxidative burst and superoxide release (Naccache *et al*., 1986 and Michel Félétou 2011).

Lipopolysaccharide (LPS), from gram negative bacteria profound effects on the host immune system (Aderem and Ulevitch 2000). The exposure of neutrophils to LPS primes the cells for enhanced release of microbial metabolites. The increase in oxidative burst might permit resistance to bacterial infection yet predispose the host to increased oxidative tissue damage and release of various inflammatory mediators. While the generated inflammatory mediators are crucial to the host defense role of the neutrophils, they can cause serve tissue damage when excessively generated. LPS-induced injury has been implicated in the pathogenesis of many diseases processes such as septic shock, myocardial infarction and adult respiratory distress syndrome (Rotnstein & Schrieiber 1988 and Roy *et al*., 2001).

Initiation of these responses depends on LPS interaction with a number of immune cells such as mononuclear phagocytes and neutrophils. The neutrophils bind the LPS- lipopolysaccharide binding proteins (LBP) complex through the CD14 receptor and thus mediate the release of a wide range of inflammatory mediators. Release of these mediators appears to be beneficial but under certain circumstances may be harmful, resulting in the systemic inflammatory response syndrome (Van Amersfoort et.al. 2003).

The present studies were undertaken to address two questions. First, if the release of arachidonic acid following stimulation of human neutrophils with PAF is potentiated in cells pre-treated with LPS and if so is this action mediated through CD14?. Second, is there a difference between the attached and suspended cells when released arachidonic acid in response to the different agonists.

MATERIAL AND METHODS

Materials:

1- Lipopolysaccharide (LPS) [Difco laboratories, USA]
2- Platelet-activating factor (PAF) [Calbiochem, USA].
3- Anti-CD14 monoclonal antibody (MY4) [coulter Corporation, USA].
4- Biocoat cell ware human fibronectin 24 well [Collaborative Biomedical products, USA].

Methods:

A. **Isolation of Human Neutrophils:** Neutrophils were isolated from normal donors using a histopaque gradient method described by (English and Anderson, 1974); then the contaminating red blood cells were lysed

by hypotonic shock (Vuorte *et al*., 2001). Heparinized (10 ml) whole blood was obtained from healthy adult donors. Neutrophils were isolated by the histopoque (LPS-free-sigma) gradient method as described by Aida and Pabst (1990). The remaining red blood cells were lysed by hypotonic shock. The neutrophils were re suspended in Hanks buffered salt solution (HBSS) without Ca^{++} or Mg^{++}. The last supernatant of cell suspension was checked for the presence of endotoxin by the limulus amebocyte lysate test.

B. **Arachidonic Acid Release in Suspended Cells as Described by (DiPersio, *et al*., 1988):** The isolated neutrophils cells were resuspended in modified HBSS, pH 7.35 containing 0.1% BSA ($1X10^7$cells/ml). Fifteen ml of the same buffer were added to 7 ml of suspended cells and the centrifuged at 1400 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 700 l of the same buffer. Specific amount (70 l) of [^{3}H] arachidonic acid was transferred into a plastic tube and dried by nitrogen gas carefully. The dried radioactive arachidonic acid was then dissolved in 700 l of the same buffer. The neutrophils cells suspension was mixed with the solution of [^{3}H] arachidonic acid and incubated at 37^{o}C for 1 hour on water- bath shaker. The cells were washed in the same buffer 3 times for 5 minutes each at 1400rpm and resuspended in 1.4ml of modified HBSS containing BSA. The suspended cells were mixed with 26.6 l of warm buffer with BSA ($2.5x10^6$cells/ml. 5 ci/ml final concentration). The cells were split into small aliquots (500 l) in small screw cap Eppendorf tubes and stimulated by different agonists (LPS, PAF OR LPS+PAF) for the appropriate time at 37^{o}C. the mixtures were centrifuged in tabletop Eppendorf 3200 centrifuge for 1 minute, the supernatant (400 l) was withdrawn and added to 3 ml of Bdget solvent (scintillation liquid) in special plastic tube (scintillation tubes). The radioactivity of these samples and control were counted using a scintillation counter.

C. Arachidonic acid release in adherent cells is released and counted as described by Ashour 1995.

Results:

1- [^{3}H] Arachidonic Acid Release in Suspended Human Neutrophils:

The [^{3}H] arachidonic acid release from prelabeled cells stimulated with LPS, LPS+PAF, serum has been measured and the results are summarized in (fig. 1).

LPS only did not produce a significant increase in [^{3}H] arachidonic acid release. However, Lps in combination with PAF produce a significant increase in [^{3}H] arachidonic acid release. The most appropriate time for the highest increase in [^{3}H] arachidonic acid release was found 45 minutes (fig. 1).

LPS did not produce a large increase a large increase in arachidonic acid release. However, LPS in combination with PAF produced produced a significant increase in arachidonic acid release. A dose response of priming of neytrophils with LPS demonstrated that 100-200 ng/ml resulted in the maximum release of arachidonic acid (fig. 2).

LPS, PAF and LPS=PAF in absence of serum caused a significant increase I arachidonic acid release as LPS and PAF in presence of serum. However, LPS+PAF in presence of serum caused a large significant increase in arachidonic acid release (fig.3).

2- [3H] Arachidonic Acid Release in Attached Human Neutrophils:

LPS alone did not produce significant increase in [^{3}H] arachidonic acid release. However, LPS+serum produced a slight increase in [^{3}H] arachidonic acid release, where LPS+SERUM+FMLP produced a significant increase in [^{3}H] arachidonic acid release. The most appropriate time for the highest increase in [^{3}H] arachidonic acid release was found at 40 minutes (fig. 4).

LPS alone, LPS with serum or LPS-FMLP did not produced a large increase in arachidonic acid release from attached cells. However, LPS in combination with serum and FLMP produced a significant increase in arachidonic acid release. It was found that a dose response a dose response of neutrophils to LPS has a maximum release of arachidonic acid with 100-200 ng/ml of LPS (fig. 5).

While MY4 has no significant effect on [^{3}H] arachidonic acid release in control, LPS, PAF OR FMLP treated cells, it inhibited greatly the potentiation by LPS-serum complex of PAF induced [^{3}H] arachidonic acid release (fig. 6).

Discussion:

Many different cell types, including neutrophils, release arachidonic acid in response to various stimuli. Neutrophils stimulated with granulocyte-macrophage colony – stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-) primes neutrophils for arachidonic acid release induced by FMLP, PAF and LTB_4 (Berry *et al*., 2008).

In response to certain infections, the human body initiates various complex reactions to fight the bacteria. These reactions activate arachidonic acid cascade and generate several biologically active substance (Markiewski and Lambris 2007).

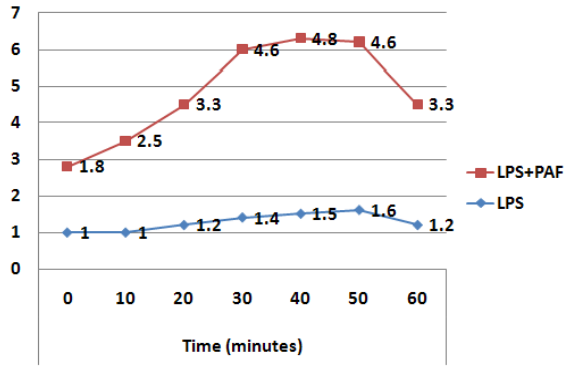

Fig. 1: Time course of suspended neutrophil priming with LPS or LPS+PAF on [^{3}H] arachidonic acid release.

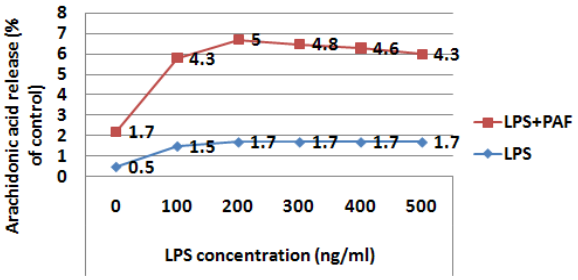

Fig. 2: Effect of LPS concentration (dose- response) on potentiation of PAF induced on [^{3}H] arachidonic acid.

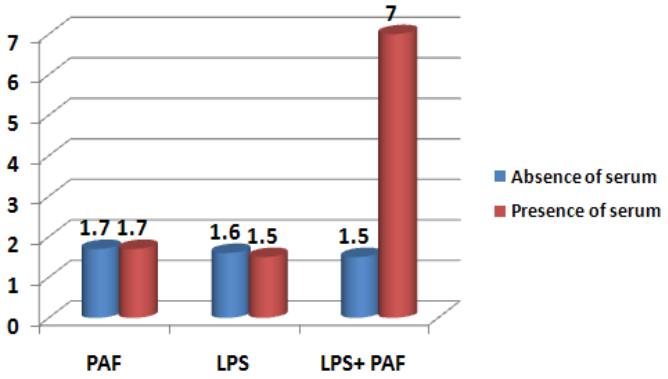

Fig. 3: Effect of absence or presence of serum on LPS potentiating of PAF induced on [^{3}H] arachidonic acid release.

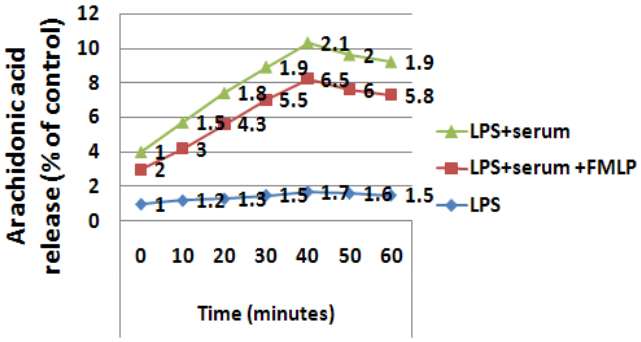

Fig. 4: Time course of attached neutrophils.

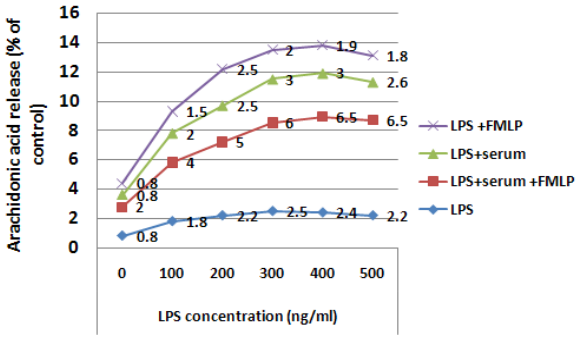

Fig. 5: Effect of LPS concentration and its potentiation on FLMP on [3H] arachidonic acid release.

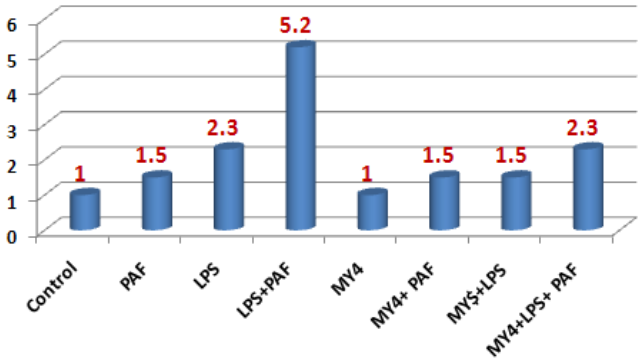

Fig. 6: Effect of anti CD14 (MY4) on LPS potentiated of [3H] arachidonic acid from neutrophils stimulated by PAF.

As shown in Fig. (1), LPS alone release arachidonic acid from suspended neutrophils but not too much as in case of LPS+PAF which caused significant increase of arachidonic acid release, and the main appropriate time was found 45 minutes. It was found also that most appropriate dose of LPS to prime neutrophils to release arachidonic acid in suspended cells was 100-200 ng/ml, and these results showed clearly that LPS alone does not cause a significant increase in arachidonic acid release where, it has a priming effect on suspended neutrophils to release [^{3}H] arachidonic acid, and there priming effects were concentration and time dependent. These results and others (DiPersio, *et al*., 1988 & Conndliffe, *et al*., 1998 and Sun *et al*., 1994) showed that the suspended neutrophils need another agonist to prime the cells for arachidonic acid release.

It was found that LPS alone reduced non significant increase in arachidonic acid release from attached neutrophils, but it primed FMLP and PAF to produce a significant increase in [^{3}H] arachidonic acid release. This [^{3}H] arachidonic acid release were concentration and time dependent, where the most appropriate time of incubation was 45 minutes and the most appropriate dose was 100-200 ng/ml. these results showed that [^{3}H] arachidonic acid was more in attached neutrophils than in suspended neutrophils.

The basis for the difference in response to the addition of GM-CSF, LPS or TNF-□ between suspended and fibronectin attached, human neutrophils is not known. These agents, unlike FMLP do not cause a rise in the intracellular concentration of free calcium when added to human neutrophils. It is generally known that attachment of human neutrophils to surfaces produce significant changes in the behavior of these cells, e.g. adherence of neutrophils to surfaces caused an increase in F-actin, a rise in the intracellular concentration of free calcium, an increase in affinity of TNF-□receptors, up regulation of adhesion molecular concentration of cyclic nucleotides (Subrahmanyam *et al*., 2001).

While MY4 has no significant effect on the [^{3}H] arachidonic acid release in control, LPS or PAF-treated cells, it inhibited greatly the potentiation by LPS-serum complex of PAF induced [^{3}H] arachidonic acid release. The experiment was repeated several times using different concentrations of MY4 and the results were almost the same. Inhibition of the potentiation was observed at an antibody concentration of 1□g/ml but the inhibition of the potentiation at 10□g/ml was greater. This means that LPS potentiates PAF-induced arachidonic acid release through CD14 receptors, where DC14 binds LPS-LBP complex on the surface of neutrophils to start the signal transduction cascade. Similar results were reported by (Le Barillec *ET AL*., 2000). It was reported that several effects of LPS (in the presence of serum) on neutrophils were inhibited by MY4 (Weingarter *et al*.,

1993). It is also established that the LPS-LBP complex binds to membrane CD14 on human monocytes. Soluble CD14 seems to play a role in the LPS- mediated activation of 14- negative cells (Wright *et al*., 1990)

The data presented in these studies made two distinctly important points:-

1-LPS or PAF alone produce small increase in arachidonic acid release in both suspended and attached human neutrophils, where LPS in combination with PAF induced significant [3H] arachidonic acid release in suspended and attached human neutrophils. This action was via CD14 receptor in neutrophils cell membrane.

2-There is a difference in response between suspended and attached neutrophils, where the attached neutrophils release more [^{3}H] arachidonic acid in response to LPS, PAF and FMLP.

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