REVIEW

Arch. Immunol. Ther. Exp., 2007, **55**, 99–110
PL ISSN 0004-069X

DOI 10.1007/s00005-007-0014-x

Regulation of the NADPH oxidase activity and anti-microbial function of neutrophils by arachidonic acid

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Received: 2006.11.13, **Accepted:** 2007.01.31, **Published online first:** 2007.03.20

Abstract

Arachidonic acid (AA), a second-messenger molecule released from membrane phospholipids by phospholipase A_2 in activated cells, is a stimulator of neutrophil responses, including the oxygen-dependent respiratory burst. The polyunsaturated fatty acid is also the precursor of biologically active eicosanoids. There are several mechanisms by which AA stimulates the respiratory burst. These include the direct binding of AA to S100 proteins which regulate the assembly of the NADPH oxidase as well as the activation of key signaling molecules which control the respiratory burst. Arachidonic acid also stimulates it own release from membrane phospholipids and this contributes to optimal respiratory burst activity. Thus, increased levels of AA at sites of inflammation will influence the magnitude and course of the inflammatory response, not only by directly affecting the function of infiltrating neutrophils and other leukocytes, but also through its metabolites generated by lipoxygenases and cyclooxygenases.

Key words: neutrophils, arachidonic acid, anti-microbial function, respiratory burst, mechanism of action.

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INTRODUCTION

Neutrophils play a major role in defense against microbial pathogens [35]. This is evident in children with cyclic neutropenia or defects in NADPH oxidase experiencing life-threatening infections [35]. The key responses involved in microbial killing are adhesion, migration and chemotaxis, upregulation of functional receptors, phagocytosis, and degranulation [35]. These events can be modulated by arachidonic acid (AA) and its metabolites [32].

AA is a polyunsaturated fatty acid which makes up cellular membranes. Typically, saturated fatty acids are esterified in the sn-1 position of the glycerol backbone of phospholipids, whereas unsaturated fatty acids such as AA are esterified in the sn-2 position. In addition to being a structural component of cellular membranes, AA is a second-messenger molecule which is released by phospholipase A_2 (PLA_2) in activated cells [7]. The liberated AA is either metabolized within the cells or is released into the extracellular space, where it can modulate the responses of surrounding cells and tissues. Thus, exogenously added AA has been reported to stimulate insulin secretion from isolated rat islets of Langerhans [8], stimulate the differentiation of HL60 cells [36] and zone chondrocytes [89], promote the adherence of human breast cancer cells to various substrata [81], stimulate the proliferation of tumor and normal cell-types [2, 101], and induce the expressions of proteins such as the mitogen-activated protein (MAP) kinase phosphatase-1 in vascular smooth muscle cells [73]. This contrasts with the ability of AA to inhibit cell--cell communication via gap junctions [3, 46], suppress the expression of adhesion molecules on cytokine-stimulated endothelial cells [98], and inhibit the proliferation of HL60 cells in conjunction with the induction of differentiation [36]. In cardiac myocytes, AA has been reported to induce intracellular acidosis [110], which could explain its inhibitory effect on the L-type Ca^{2+} channel [83] and Ca^{2+} transients [50], and inhibit the ATP-sensitive K^+ channel but activate the ATP-insensitive K^+ channel with an outward rectifying property

[56]. Although the metabolism of AA by lipoxygenases and cyclooxygenases yields biologically active eicosanoids [38, 84], many of the above actions of AA are independent of the formation of these metabolites. Recent evidence from studies in leukocytes have demonstrated that AA can even regulate its own metabolism via the 5-lipoxygenase [19, 37].

PLA_2 AND THE GENERATION OF NON-ESTERIFIED AA

Although AA is present in the plasma and interstitial fluid, this level is generally insufficient to cause any significant biological responses. It is the pool which is released by PLA_2 during cell activation, tissue injury, or in an inflammatory reaction which is believed to be responsible for exerting the observed biological actions.

PLA_2 cleaves the ester bond at the sn-2 position of phospholipids, thereby liberating the esterified fatty acid and a lysophospholipid. Currently, 14 groups which comprise a number of subgroups of PLA_2 have been described [7]. These include the four main types of secretory PLA_2 ($sPLA_2$), which comprises a family of related enzymes [105], cytosolic PLA_2 ($cPLA_2$), of which there are two paralogues, α and β [7], Ca^{2+}-independent PLA_2 ($iPLA_2$), and platelet-activating factor (PAF) acetyl hydrolase [7, 95]. Generally speaking, while $cPLA_2$ shows a preference for AA at the sn-2 position, $sPLA_2$ and $iPLA_2$ do not [7, 105]. Accumulating evidence suggests that some forms of $sPLA_2$ can act in concert with $cPLA_2$ to induce eicosanoid formation and biological responses [5, 58]. For example, it has been demonstrated that exogenously added human group V $sPLA_2$ can cause the release of AA and leukotriene B_4 (LTB_4) from human neutrophils by activating $cPLA_2$ via an increase in cellular Ca^{2+} concentration and $cPLA_2$ phosphorylation [57, 58]. In mouse peritoneal macrophages it has recently been demonstrated that upon ingestion of zymosan, group V $sPLA_2$ is recruited to the phagosome, where it co-localizes with $cPLA_2$, 5-lipoxygenase, 5-lipoxygenase-activating protein, and leukotriene C_4 synthase [5]. In addition, there is evidence in leukocytes and other cell-types that there is a sequential activation of $cPLA_2$ and $sPLA_2$ following cell activation [6, 85]. Several murine lines in which a form of PLA_2 such as group V $sPLA_2$ or $cPLA_2$ is deleted have been used to define the roles these forms play. For example, studies in mice in which the group V $sPLA_2$ is deleted have demonstrated that this $sPLA_2$ species contributes to the innate immune response both through regulation of eicosanoid generation in response to a phagocytic stimulus and also as a component of the phagocytic machinery [5]. Thus it was demonstrated that the ability of peritoneal macrophages from group V $sPLA_2$-deficient mice to phagocytose opsonized zymosan was reduced by 50% compared with cells from wild-type animals [5]. A similar role for $cPLA_2$ in innate immunity has been proposed. Thus, neutrophils from cPLA2α-deficient mice have a reduced capacity to kill *E. coli in vitro* and this is accompanied by a reduced clearance of *E. coli in vivo* [87].

The liberated AA has several fates. It can be released into the extracellular space or metabolized to eicosanoids. Alternatively, the liberated AA can act as a second-messenger molecule within the cell to stimulate or modulate the activities and function of signaling molecules such as protein kinase C (PKC) [45, 51] and rhoGDI [22], a guanine nucleotide inhibitor of rho, a member of the small G protein family. In the extracellular space, the fatty acid and metabolites can exert their effects in an autocrine or exocrine manner.

CONCENTRATIONS OF AA WITHIN CELLS AND IN THE PLASMA

What concentrations of AA are required to evoke cellular responses and can these levels be achieved intra- or extracellularly? Although some studies have tested AA at concentrations of 100 μM and above [4, 26], concentrations of 5–30 μM [32] are typically sufficient to elicit a response. However, there are also reports of biological actions at below 5 μM [78]. The lower concentrations, up to 30 μM, are likely to be achievable under physiological and pathophysiological conditions. For example, in glucose-stimulated rat islets of Langerhans, the intracellular concentration of free AA has been reported to be in excess of 20 μM, rising from undetectable levels in unstimulated islets [108]. In activated neutrophils, the intracellular free AA has been reported to exceed 800 pmol/10^7 cells, compared with undetectable levels in resting cells [107]. We estimate this to be around 57 μM, assuming that the average diameter of a neutrophil is 14 μm and an even distribution within a spherical volume of approximately 1.4 pl. Another study has found neutrophils to contain 100–2200 pmol/10^7 cells [77]. Our own studies in tumor necrosis factor (TNF)-stimulated neutrophils have found these cells to contain 125 pmol/10^7 cells of AA compared with 15 pmol/10^7 in unstimulated cells [86].

In the plasma, about 6–12% of all non-esterified fatty acid (NEFA) is AA [52, 106]. Data from the emergency clinic have shown that the circulating levels of NEFA following myocardial infarction can exceed 1.5 mM [102], compared with 0.2–0.68 mM in patients presenting at the emergency ward for non-cardiac related complaints [62]. Although most of the NEFA in the circulation is bound by proteins such as albumin under physiological conditions, a large increase in circulating NEFA following on acute pathological condition could result in large amounts of NEFA becoming available for uptake by tissues [80]. Thus when the fatty acid:albumin molar ratio exceeds 2, occurring at about 1.2 μM of circulating NEFA [80], exponential uptake of NEFA by tissues can be expected [94].

The levels of NEFA, including AA, are increased in infarcted rat and murine hearts and further increases

occur during reperfusion [24, 30]. In ischemic-reperfused hearts, the increase in AA is between 8- and 23--fold [24, 30]. Increases in the levels of AA and other NEFA have also been reported in other ischemic tissues, such as the brain [13]. While catacholamines have been suggested to cause the mobilization of AA from adipose tissues during ischemic conditions [80], the rise in AA in the ischemic myocardium has been reported to be due, predominantly, to the action of a Ca^{2+}-independent PLA_2 [66].

Increases in the ambient concentrations of AA are also observed in other pathological states. Thus the levels of AA have been reported to be increased in cerebral malaria [111]. The above studies therefore strongly suggest that the amount of AA within activated cells or in the tissues under pathological conditions can reach the levels that are used under various experimental conditions.

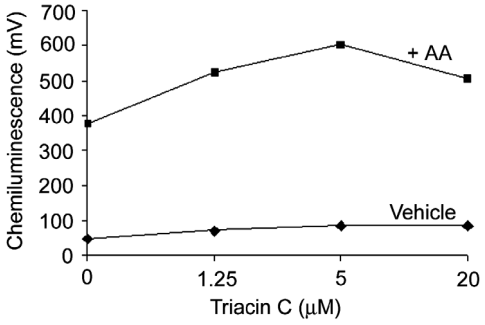

Fig. 1. Esterification of AA into membrane phospholipids is not required for the stimulation of neutrophil respiratory burst. Neutrophils were pretreated with triacin C for 10 min before being incubated with AA (20 μM). The respiratory burst activity, measured as lucigenin-detected production of superoxide, was monitored and the peak rate of chemiluminescence is recorded and shown.

DO FATTY ACIDS NEED TO ENTER THE CELLS TO ACT AND WHAT MECHANISMS ARE INVOLVED IN THE UPTAKE OF AA?

While no receptors have been reported for intercepting AA, a number of membrane proteins have been described which can bind AA and other fatty acids. In the main, these are involved in transporting the fatty acids across the plasma membrane [59]. However, we and others have reported that members of the epidermal growth factor receptors may be involved in mediating some of the effects of AA [1, 49]. Thus we previously demonstrated that blocking $ErbB_4$ in human umbilical vein endothelial cells with tyrosine kinase inhibitors with selectivity for Erb receptors inhibited the ability of AA to stimulate the activity of class 1a phosphatidylinositol 3 kinase (PI3K) [49]. Apart from this, very little is known about the initial stages of cell activation by not only AA, but also other fatty acids. It is likely that other effects of AA would require its entry into the cells to access intracellular sites.

Our studies with AA in human neutrophils have demonstrated that exogenously added AA is rapidly taken up by the cells and esterified in membrane phospholipids [86]. However, esterification is not always required for biological activity. The data in Fig. 1 demonstrate that the addition of triacin C, an inhibitor of long-chain acyl synthetase [60], does not prevent AA from stimulating neutrophil superoxide production. Being lipophilic, it is generally assumed that fatty acids can traverse the plasma membrane by diffusion. Whereas the uptake of short-chain fatty acids is predominantly via a passive diffusion process [53], there is still much controversy regarding the mechanism(s) of uptake of long-chain fatty acids (14 to 22 C atoms). Whether this is through passive diffusion driven by the concentration gradient across the plasma membrane [53] and/or mediated by protein carriers [97] remains a hotly debated question. There is a substantial amount of evidence from cell types such as hepatocytes, adipocytes, and muscle cells/cardiomyocytes or from membrane models in support of both modes of transport. These issues are discussed in detail in excellent reviews by Koonen et al. [59], Stahl [97], and Kamp and Hamilton [53].

A common finding in many studies on the biological actions of exogenous fatty acids is the ability of delipidated serum albumin to rapidly terminate the actions of these fatty acids [3, 46]. This is attributed to the ability of the delipidated albumin to "extract" the fatty acids from the plasma membrane and perhaps the cell interior. Such a rapid termination of the effects of AA suggests that the fatty acid may be located in the outer leaflet for biological activity or that there is a very rapid reverse transport/diffusion from the cell interior to the outer leaflet and that the native and NEFA, not a metabolite, is responsible for the biological action [10, 12].

AA AND NEUTROPHIL ANTI-MICROBIAL ACTIVITY

Our previous studies have demonstrated that AA increases the ability of the neutrophils to kill microorganisms. For example, AA enhanced the killing of the asexual blood forms of *Plasmodium falciparum* by neutrophils [61]. This parasite killing effect of AA was not affected by inhibitors of lipoxygenases or cyclooxygenases. At an optimal fatty acid concentration, this effect exceeded that caused by TNF [61]. At suboptimal concentrations, AA and TNF caused a synergistic increase in neutrophil-mediated parasite killing. These data suggest that AA is able to upregulate the mechanisms involved in microbicidal activity. Consistent with this notion, the addition of AA to isolated neutrophils results in the stimulation of adherence to various substrata, superoxide production, degranulation, and a num-

ber of other responses [35]. However, inhibitory effects are also observed. Thus, AA is a powerful inhibitor of neutrophil migration [34]. These findings suggest that at the site of an inflammatory response, such as tissue injury, the recruited neutrophils are stimulated by AA, produced by surrounding activated or injured tissues and infiltrating leukocytes, in a range of microbicidal responses and are prevented from leaving such sites until resolution occurs. At these sites, AA is also metabolized to the various eicosanoids. Whereas some metabolites are pro-inflammatory, others such as lipoxin A_4 (LXA_4) have anti-inflammatory activity and hence have been proposed to play a role in the resolution of the inflammatory response [38, 90]. These issues will be discussed further below.

Historically, the first reported effect of AA on neutrophil responses was on neutrophil chemotaxis. In this study, AA was found to stimulate neutrophil chemotaxis [104]. However, this was not substantiated by subsequent studies [82], including ours. We demonstrated that AA totally blocked the random migration and chemotactic response to formyl Met-Leu-Phenylalanine (fMLP) [34]. This effect of the fatty acid was dependent on its concentration, with a threshold at approximately 10 μM. Structure-function analysis demonstrates that a free carboxyl group is required for this inhibitory effect. Hydroxylated derivatives were less effective than the native AA. The inhibitory effects were not affected by inhibitors of cyclooxygenases or lipoxygenases [34].

Following the initial reports on the effect of AA on chemotaxis, AA was subsequently demonstrated to stimulate neutrophil aggregation [79]. In this study, cytochalasin B, a potentiator of neutrophil responses, enhanced the AA-induced aggregation, but the response was blocked by 5,8,11,14-eicosatetraynoic acid, an inhibitor of AA metabolism. Aggregation of neutrophils was not stimulated by several fatty acids with structural similarity to AA. These results suggest that metabolic derivatives of AA may be active in stimulating aggregation. Two of these have been identified to be 5--hydroperoxyeicosatetraenoic acid and its derivative, hydroxyeicosatetraenoic acid [20]. This is one response which has been attributed to AA metabolites.

Although early studies failed to observe an effect of AA on neutrophil degranulation, even in the presence of cytochalasin B [79], others have reported that AA is also a secretagogue of rabbit peritoneal neutrophils [76]. Our studies have demonstrated that AA is a complete neutrophil secretagogue, stimulating the degranulation of both the specific and azurophilic granules [10]. This occurs over a concentration range of 10–33 μM. As discussed in the above sections, these concentrations are likely to be within the ranges found in activated cells.

AA is a stronger stimulator of neutrophil degranulation than the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) or the classical neutrophil activator fMLP [33]. This action of AA is consistent with its ability to promote the fusion between isolated neutrophil-specific granules and liposomes by lowering the requirement for calcium in this process [15]. Although LTB_4 has been reported to stimulate neutrophil degranulation [16], our studies demonstrated that the effect of AA on degranulation was not blocked by inhibitors of lipoxygenases or cycloxygenases [12]. In fact, introduction of a hydroperoxy or hydroxyl group abolished the stimulatory effect of AA [12].

Stimulation of neutrophil respiratory burst by AA is one of the best characterized effects of the fatty acid on neutrophil responses. Upon stimulation, neutrophils undergo a respiratory burst which results in the production of superoxide by the multi-component NADPH oxidase [25]. This enzyme system is composed of the soluble components p40phagocyte oxidase (phox), p47phox, p67phox, rac 2, and rap1a and the membrane--bound components gp22phox and gp91phox, which make up flavocytochrome b558 [25]. Recent studies have also implicated members of the S100 proteins as components of the NADPH oxidase (see below). Since the studies by Badwey et al. in 1981 [4], which showed that AA and other unsaturated fatty acids can stimulate superoxide production in intact neutrophils, many groups have extended these findings. This response is similar in magnitude to that observed with PMA [40], which is several times greater than the response seen with fMLP. The kinetics of the AA-stimulated production of superoxide lags behind that of fMLP and is generally detectable within 0.5 min and reaches a maximum at 2–2.5 min [40]. Dose-response studies in intact cells have demonstrated that this response is detectable at approximately 5 μM and increases with increasing concentrations of AA [40]. The stimulatory effect of AA on intact neutrophils can be reproduced in cell-free systems, provided that all the components of the NADPH oxidase are present [72]. As discussed above (Fig. 1), the stimulatory effect of AA on superoxide production is not dependent on its esterification into membrane phospholipids since this effect is not affected by the addition of triacin C.

An interesting property of AA is its ability to prime neutrophils for an enhanced respiratory burst. Thus we have previously reported that pretreatment of neutrophils with low concentrations of AA primed the neutrophils for an enhanced respiratory burst in response to a subsequent challenge with TNF [63]. As discussed below, this effect is likely to have been caused by the upregulation of surface TNF receptors by the fatty acid.

The adherence of neutrophils to its targets is an essential step in the microbicidal activity. As mentioned above, AA promotes the adherence of neutrophils to plasma-coated surfaces and this effect is comparable in magnitude to that observed with TNF or fMLP [10]. Again, this effect is not altered by inhibitors of lipoxygenases or cyclooxygenases, suggesting that AA itself is the active compound. One way in which AA is able to promote the adherence of neutrophils is via the upregulation of surface receptor expression. Thus, not surprisingly, AA enhances the expression of the β2 integrin CD11b/CD18 on the surface of neutrophils [11, 33]. This

effect could be observed over an AA concentration range of 5–80 μM [11]. Such an effect could contribute to the AA-stimulated adherence of neutrophils to various substrata, including endothelial cells [10, 11].

The increase in the expression of CD11b/CD18 and other receptors caused by AA suggests that these changes on the surface of the neutrophils are likely to be responsible for priming the neutrophils for an enhanced microbicidal activity, not only in relation to components of the microorganisms, but also to pro-inflammatory mediators produced by the host. Consistent with this, we reported that AA can upregulate the expressions of both the p55 and p75 TNF receptors on neutrophils, but suppress the expressions of these receptors in other cell--types [74]. This neutrophil-specific effect is restricted to the mature cells, since this upregulation in TNF receptor expression could only be reproduced in differentiated, but not in undifferentiated HL60 cells [74]. Dose--response studies have demonstrated that this response occurs at 5 μM and above. The effect of AA was not dependent on the formation of metabolites, since inhibitors of lipoxygenases and cyclooxygenases did not block this effect of AA. Indomethacin, a non-selective cyclooxygenase inhibitor, actually increased the expression of TNF receptors [74], whereas expression was down-regulated by LTB_4 [88]. The increase in surface TNF receptors is likely to be the result of recruitment from neutrophil granules, which are known to contain "spare" TNF receptors, following AA-stimulated degranulation. Consistent with the increased expression of TNF receptors, AA-pretreated cells exhibit enhanced capacity to produce superoxide in response to a subsequent challenge with TNF [74]. These data suggest that AA may promote the inflammatory response by up-regulating the expression of TNF receptors, which makes these cells more responsive to the cytokine.

ROLE OF PLA_2 IN NEUTROPHIL SUPEROXIDE PRODUCTION

Current evidence suggests that neutrophils express $sPLA_2$ [28], $cPLA_2$ [70, 85], and $iPLA_2$ [103]. Although neutrophils have been reported to synthesize PAF [68], which is formed by the PLA_2-like PAF acetyl hydrolase, this enzyme has received little attention [95].

Early evidence for a role of PLA_2 in regulating neutrophils responses came mainly from two types of studies. The first involved preincubating neutrophils with labeled AA and showing that labeled AA was released when the cells were stimulated. This is complemented by investigations which examined the effects of inhibiting the enzyme and or reducing the expression of the enzyme. These studies demonstrate that $cPLA_2$ is activated upon the binding of ligands, such as fMLP [70], opsonized zymosan [42], and TNF [86], to their respective receptors. Studies in a myeloid phagocytic cell line have demonstrated that incubation of these cells with fMLP or zymosan results in the interaction of $cPLA_2$ with p47phox, p67phox, and gp91phox [91]. This agonist--stimulated interaction between components of NADPH oxidase and $cPLA_2$ appears to facilitate the translocation of phosphorylated $cPLA_2$ to the plasma membrane, in addition to its known localization to the perinuclear region. In cells which are deficient in gp91phox, $cPLA_2$ fails to translocate to the plasma membrane [91]. Associated with the translocation of $cPLA_2$ to the plasma membrane is enhanced production of superoxide by the cells. On the other hand, studies in activated human monocytes demonstrated that $cPLA_2$ is required for the translocation of p47phox and p67phox to the plasma membrane [114]. Thus, depletion of $cPLA_2$ by antisense oligodeoxyribonucleotide specific for $cPLA_2\alpha$ mRNA prevented ligand-stimulated translocation of p47phox and p67phox, an effect which can be overcome by the addition of exogenous AA [114]. Depletion of $cPLA_2\alpha$ also inhibited ligand-stimulated superoxide production. The reason for the above discrepancy is not known, but could reflect cell-type differences. Other studies have found that pharmacological inhibitors of $cPLA_2$ blocked ligand-stimulated superoxide production [42], consistent with a positive role for $cPLA_2$ in this response. Recently, a role for $cPLA_2$-derived AA in the enhancement of neutrophil superoxide production by advanced glycation end (AGE) products, formed under hyperglycemic conditions in diabetics, has also been reported [109]. Methyl arachidonyl fluorophosphonate, a specific $cPLA_2$ inhibitor, abrogated the AGE-augmented superoxide production, whereas inhibitors of $sPLA_2$ and $iPLA_2$ did not. However, methyl arachidonyl fluorophosphonate did not affect the production of superoxide in response to fMLP or mechanical stimulation of the neutrophils [109], thereby questioning the role of $cPLA_2$ in fMLP-stimulated neutrophils. In support of this argument, a recent study with $cPLA_2\alpha$-deficient mice has also questioned the role of $cPLA_2$ in the regulation of superoxide production and a number of other responses. Although the lack of $cPLA_2\alpha$ impaired the *in vitro* killing of bacteria by neutrophils and *in vivo* clearance of *E. coli* from the lungs [87], there was no reduction in the ability of $cPLA_2\alpha$-deficient neutrophils to produce superoxide in response to PMA, fMLP, or zymosan [87]. Degranulation in response to the Ca^{2+} ionophore ionomycin, phagocytosis of bacteria, and ligand-stimulated expression of CD11b were also not affected. In these $cPLA_2\alpha$-deficient neutrophils there was no detectable release of AA following activation. The authors concluded that $cPLA_2\alpha$ is not essential for neutrophil superoxide production, degranulation, or phagocytosis even though the enzyme is involved in the innate immune response to bacterial infection *in vivo*. It was argued that the impairment in microbicidal activity was due to an impairment in phagocytosis-independent bacterial killing [87], such as that mediated by neutrophil extracellular traps [17]. Interestingly, the decrease in the *in vitro* bactericidal activity could be rescued partially by the addition of AA, but not LTB_4 [87]. This demonstrates that the killing process is still depen-

dent on AA. There are perhaps other possible explanations for the surprising data from the $cPLA_2\alpha$-deficient neutrophils. For example, the loss of $cPLA_2\alpha$ may be compensated by $sPLA_2$ or $iPLA_2$, which do not show any preference for AA at the sn-2 position. Since other PUFA can mimic the actions of AA [35], responses such as superoxide production, degranulation, and phagocytosis may proceed at the normal rate. There is some evidence in human neutrophils which shows that $iPLA_2$ activity can be coupled to superoxide production, independently of $cPLA_2$ [103]. The role of $cPLA_2\alpha$ in ligand--stimulated superoxide production, degranulation, and phagocytosis therefore awaits further clarification.

Interestingly, we have demonstrated that AA can stimulate the activity of $cPLA_2$, which is then followed by the activation of $sPLA_2$ [85]. AA does not affect the activity of $iPLA_2$ [85]. Using inhibitors to inhibit either $cPLA_2$ or $sPLA_2$, the data demonstrate that both are involved in regulating AA-stimulated superoxide production.

HOW DOES AA STIMULATE NEUTROPHIL SUPEROXIDE PRODUCTION?

The ability of high concentrations of AA (e.g. 100 μM) and detergents such as sodium dodecyl sulfate to cause the production of superoxide in either intact [4, 26] or cell-free systems prepared from soluble and membrane fractions of neutrophils [23, 69] has prompted the suggestion that AA acts in a detergent-like manner. However, a number of observations in intact cells argue against this notion, especially at the lower concentrations which our laboratory and others have used (<30 μM). It is highly likely that a number of mechanisms are involved in causing the activation of NADPH oxidase. Some may regulate the translocation of cytoplasmic components, whereas others could regulate the assembly of the multi-components at the plasma membrane.

AA has been reported to stimulate the activities of a range of intracellular signaling molecules in neutrophils. These include PKC [47, 51, 71], members of the MAP kinases such as p38 and the extracellular signal regulated protein kinases (ERK)1 and ERK2 [45, 47], and class 1a PI3K [49]. In addition, AA has been reported to stimulate the mobilization of stored Ca^{2+} [41] and the activities of $cPLA_2$ and $sPLA_2$ [85] and to open a H^+-selective channel on the plasma membrane [65]. Our data and those of others have demonstrated that the stimulation of superoxide production by the PUFA is mediated, at least in part, via PKC, p38, ERK1 and ERK2, and PI3K. Thus, pharmacological inhibitors used at concentrations which show specificity for their intended targets have demonstrated that the ability of AA to stimulate superoxide production can be inhibited by GF109203X, SB203580, PD98059, and wortmannin, inhibitors of PKC, p38, the MAP kinase ERK kinase (MEK)-ERK modules, and PI3K, respectively [35]. When neutrophils were treated at suboptimal doses of a combination of GF109203X, SB203580, and PD98059, AA-stimulated superoxide production was inhibited in an additive manner. Interestingly, we have reported that in addition to AA being able to stimulate the activities of $cPLA_2$ and $sPLA_2$ in neutrophils, our data demonstrate that the ability of AA to stimulate the respiratory burst can be inhibited by inhibitors of $cPLA_2$ and $sPLA_2$ [85]. In contrast, the stimulation of superoxide production by AA in cell-free systems is not inhibited [69], suggesting the involvement of another mode of action. These findings not only argue against a non-specific membrane-perturbing or detergent effect being involved in the action of AA in intact neutrophils, but also that AA is a constituent of a signaling loop in which the fatty acid regulates its own production and function. This aspect will be discussed in more detail below.

Consistent with a role for PKC and the MAP kinases, both p47phox and p67phox have been reported to be phosphorylated by PKC, ERK1/ERK2, and p38 [27, 29]. Such phosphorylation may regulate the translocation of the cytoplasmic components to the plasma membrane [27, 29]. Interestingly, structural analysis of p40phox, p47phox, and p67phox have revealed that they each contain a domain which binds PtdIns 3 P and PtdIns 3,4 P_2 [54, 96, 113], both of which are formed following the activation of PI3K. This raises the interesting possibility that the inhibition of AA-stimulated neutrophil superoxide production by PI3K inhibitors results from reduced interaction between p47phox or p67phox with PtdIns 3 P and PtdIns 3,4 P_2, the formation of which is blocked by the inhibitors.

In addition to the requirement for signaling molecules in its action, there are at least two other complementary regulatory steps through which AA stimulates the respiratory burst. The first involves the direct binding of AA to the S100 proteins S100A8 and A9 (also known as MRP 8 and 14, respectively) [92], which are abundantly expressed in neutrophils. Acting as a complex, S100A8 and S100A9 bind calcium, which is a prerequisite for binding AA [93]. Deletion and point mutation studies have implicated H103, H104, and H105 in the C-terminal tail of S100A9 as being crucial for binding the carboxyl group of AA [93]. Recent studies have implicated the S100 proteins as components of the NADPH oxidase. Thus it has been demonstrated that the S100 proteins interact with members of the NADPH oxidase, predominantly p47phox, p67phox, and rac 2 [31, 55], and they translocate to the plasma membrane in a gp91phox-dependent manner. Thus, binding to the S100 proteins would explain how AA is able to stimulate superoxide production in cell-free systems with neutrophil extracts and hence argues against the notion that AA mimics a detergent.

During the activation of the NADPH oxidase, a single electron is transferred from internal NADPH, through the oxidase complex, to external oxygen. There is a rapid depolarization of the membrane potential from –60 mV to –15 mV and a slight (0.1) fall in pH [43]. This creates an imbalance in charge across the plasma

membrane. To compensate for this charge difference, efflux of H^+ ions through a channel occurs and AA has been reported to play a role in opening this channel. In the resting neutrophil, the H^+ channel is closed and the opening is associated with the initiation of superoxide production induced by a stimulus. Studies with neutrophil cytoplasts which lack the nucleus, granules, and other internal organelles, have demonstrated that the channel can be opened by AA and not by depolarization of the plasma membrane, a change in the pH gradient, or a combination of the two [44]. The direction and extent of H^+ flux is dictated by the proton motive force. It appears, therefore, that AA is required for the production of superoxide and efflux of H^+. A role for $cPLA_2$ in opening the H^+ channel has been reported [65, 67, 99].

ROLE OF METABOLITES OF AA

With the exception of AA-stimulated neutrophil aggregation [79], the above-listed effects of AA on neutrophil responses are not dependent on metabolites of the cyclooxygenases or lipoxygenases since inhibitors of these enzymes do not block these actions. These data imply that AA can promote the inflammatory reaction by exerting a direct effect on neutrophils, independently of its metabolites, which are also able to stimulate neutrophil responses. However, as described above, biologically active metabolites of AA are formed by neutrophils. There are a number of in-depth reviews on the metabolism of AA and the effects AA metabolites have on these leukocytes [84, 90]; the reader is referred to these for a more detailed discussion.

In neutrophils, AA is predominantly metabolized by the 5-lipoxygenase pathway to yield the 4-series leukotrienes, such as LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE), the source of 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) [84]. Previously reported actions of 5-HETE on neutrophil responses are now attributed to 5-oxo-ETE. The production of 5-oxo-ETE, catalyzed by a 5-hydroxyeicosanoid dehydrogenase, requires $NADP^+$ and is stimulated by the activation of the respiratory burst and oxidative stress, suggesting that this metabolite acts predominantly under conditions of oxidative stress. Following cell stimulation, LTB_4 and 5-oxo-ETE are produced with similar kinetics [84]. Thus, both LTB4 and 5-oxo-ETE are able to stimulate neutrophil integrin expression, degranulation, and Ca^{2+} mobilization which is due to their binding to their respective G-protein-coupled receptors [84]. While LTB_4 acts predominantly via the high-affinity BLT1 [112], the receptor for 5-oxo-ETE has not been identified. Both metabolites stimulate actin polymerization and are strong chemoattractants for neutrophils [84], in direct contrast to AA, which is a powerful inhibitor of neutrophil migration [34]. LTB_4 is a stimulator of neutrophil superoxide production, whereas 5-oxo-ETE has not been consistently reported to stimulate superoxide production [84]. In general, LTB_4 is 5–10 times more potent that 5-oxo-ETE in stimulating neutrophil responses. However, when the neutrophils are primed with cytokines such as TNF-α or granulocyte-macrophage colony stimulating factor, the responses to 5-oxo-ETE are enhanced to the levels observed with LTB_4 [84]. Owing to these differences and the conditions which favor the production of 5-oxo-ETE, it is likely that LTB_4 is a more important metabolite than 5-oxo-ETE in the initial stages of inflammation. In persistent inflammatory conditions and with increasing oxidative stress, the LTB_4 receptors become desensitized owing to homologous desensitization and 5-oxo--ETE becomes the dominant active metabolite, especially in the milieu of increasing levels of pro-inflammatory cytokines. The higher metabolic stability of 5-oxo-ETE over LTB_4,which is rapidly metabolized by ω-oxidation, lends further support to this idea.

Another AA metabolite which has a profound effect on neutrophils is LXA_4. This metabolite is formed by a transcellular mechanism involving the 5-lipoxygenase of one cell-type and the 15-lipoxygenase of another cell--type [90]. *In vivo* it has been demonstrated that the production of LXA_4 coincides with that of LTB_4 [9]. In the presence of aspirin, an analogue, 15-epi-LXA_4, is formed [90]. These metabolites exert anti-inflammatory effects on neutrophils by inhibiting their activation and chemotaxis [90]. LXA_4 acts via a G-protein-coupled receptor, ALX [21], which has high sequence homology to members of the family of fMLP receptors, and its binding to the low-affinity fMLP receptor, FRPL1, has prompted some to consider this to be the LXA_4 receptor as discussed by Chiang et al. [21]. Because of its inhibitory effect on neutrophil chemotaxis, this metabolite has been proposed to play an important role in the resolution of acute inflammatory response.

CENTRAL ROLE OF AA IN A SIGNALING LOOP

We suggested previously that AA liberated through the activation of the $cPLA_2$ could amplify or prolong intracellular signaling initiated by a receptor that is coupled to the enzyme [49]. In this scenario, the binding of an agonist to its receptor results in the activation of signaling molecules, such as $p21^{ras}$, PKC, ERK1/ERK2, p38, PI3K, and Akt. Activated ERK1/ERK2, and/or p38 phosphorylate $cPLA_2$ [39, 64], and if the intracellular Ca^{2+} concentration is also elevated after receptor engagement, phospholipid hydrolysis by $cPLA_2$ occurs. The liberated AA is released into the extracellular space which can act in an autocrine manner, thus setting up a signaling loop (Fig. 2). The demonstration that exogenous AA increases the concentration of intracellular Ca^{2+} and stimulates the activities of PKC, ERK1/ERK2, p38, $cPLA_2$, $sPLA_2$, and PI3K [45, 47, 49, 85] is consistent with such a loop. Current evidence, although incomplete, suggests that such a loop may exist, at least

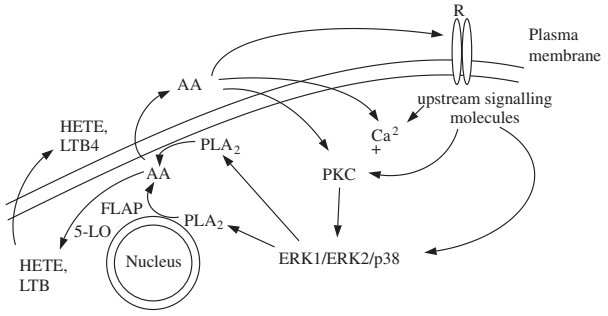

Fig. 2. Signaling loop involving AA**.** Activation of appropriate receptors on the plasma membrane results in the activation of PKC and MAP kinases and elevation in the concentration of intracellular Ca^{2+}. This results in the activation of $cPLA_2$ and $sPLA_2$, which release AA and other fatty acid. While some of the AA is metabolized by the lipoxygenases and cyclooxygenases, a proportion is released extracellularly. This can act in an autocrine or paracrine manner to either prolong or amplify the effect of receptor activation via the activation of PKC and MAP kinases and elevation of intracellular Ca^{2+} levels, thus forming a positive feedback loop. Functionally, this loop can contribute to the overall response of the cell to receptor stimulation. A subsequent increase in the expression of MAP kinase phosphatases (MKP) such as MKP-1 provides a termination signal to the loop. Abbreviations are: R – receptor, PKC – protein kinase C, ERK – extracellular signal-regulated protein kinase, PLA_2 – phospholipase A_2, 5-LO – 5-lipoxygenase, FLAP – 5-LO-activating protein, HETE – hydroxyeicosatetraenoic acid, LTB4 – leukotriene B4.

in human neutrophils. Thus, ligation of the fMLP or FcγRIIa or FcγRIIIb receptors results in the activation of all the above-listed signaling molecules and the liberation of AA [42, 100]. To further prove the existence of the loop, it is also essential to demonstrate that inhibition of $cPLA_2$ decreases the magnitude of receptor-mediated activation of signaling molecules that are upstream of $cPLA_2$, such as the MAP kinases. Although evidence for this in neutrophils is still lacking, studies in human monocytic cells have demonstrated that inhibition of either the activity or the expression of $cPLA_2$ resulted in the attenuation of ligand-stimulated activation of MAP kinases [18]. The observations that inhibitors of PLA_2, the ERK or p38 modules, inhibit not only fMLP- [48, 100], but also AA- [35, 85] stimulated superoxide production suggest that this positive feedback loop is functional in neutrophils. The idea of positive feedback signaling loops involving signaling molecules such as PKC, ERK1/ERK2, and PLA_2 has been the subject of a modeling study [14]. Depending on the strength and duration of the initial signal at the cell surface, the model predicts that signaling via these loops can become self-sustaining, and this is supported by our observation that exogenous AA stimulates the activities of $cPLA_2$ and $sPLA_2$ [85]. The model proposes that termination of this signaling loop is facilitated by a MAP kinase-mediated increase in the expression of MAP kinase phosphatase-1, a dual-specificity phosphatase which dephosphorylates and inactivates the MAP kinases [14]. Interestingly, AA has been reported to induce the expression of MAP kinase phosphatase-1 [73], and this provides a plausible mechanism for terminating signaling via this loop.

CONCLUDING REMARKS

Emerging evidence demonstrates that apart from being an integral structural component of membrane phospholipids and other lipid pools, a source of energy and a substrate for the lipoxygenases and cyclooxygenases, AA is also a signaling entity *per se*. Non-esterified AA is found intracellularly and extracellularly in biological fluids during cell activation and inflammation. There is strong evidence that AA regulates neutrophil functions by employing a variety of intracellular signaling molecules. These functions are critical to microbial killing by the leukocytes. Since the levels of AA in membrane phospholipids are in continuous flux, modulation of immune responses may not be readily evident, but variations caused by changes to the intake of dietary fatty acids could be important in determining susceptibility to infection or increased tissue damage in autoimmune inflammatory disorders. This knowledge will lead to new approaches and pathways to target in inflammatory and infectious diseases.

Acknowledgment: The authors are indebted to Dr. B. S. Robinson, who conducted the experiments on Triacin C. The studies from the authors' laboratory were supported by funds from the National Health and Medical Research Council of Australia, National Heart Foundation of Australia, Women's and Children's Hospital Research Foundation, and Channel 7 Children Research Foundation.

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