Local Anesthetic Effects on Priming and Activation of Human Neutrophils

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Background: Local anesthetics (LAs) have been shown to inhibit human polymorphonuclear neutrophil (hPMN) functions in vitro, but mechanisms are poorly understood. In this study the authors determined how LAs affect superoxide anion production of hPMNs primed with platelet-activating factor (PAF). The authors studied which pharmacologic properties of LAs are important for this action and assessed the LA site of action within the PAF signaling pathway.

Methods: Metabolic activity of primed and/or activated hPMNs were measured using the cytochrome-c assay. hPMNs were incubated with several LAs for 1 h to assess interference with PAF signaling. Using protein kinase C (PKC) inhibitors, the PKC activator phorbol myristate acetate (PMA), and the phospholipase C (PLC) antagonist U-73122, we studied involvement of PKC and PLC in the priming process. Pertussis toxin (PTX) was used to characterize the G proteins mediating this pathway. Combined administration of lidocaine with PMA or PTX was used to determine the LA site of action within the priming pathway.

Results: Platelet-activating factor effectively primed hPMNs. Ester LAs (tetracaine and benzocaine) exerted the most profound inhibitory effect on PAF-primed hPMNs, whereas inhibitory potency of amide LAs increased with decreased charged fraction. The major PAF-induced priming pathway is PLC- and PKC-dependent and mainly Gq-mediated. The main target site for LA in this pathway is located upstream of PKC.

Conclusions: Local anesthetics in clinically relevant concentrations inhibit superoxide anion production of PAF-primed hPMNs. Effects on priming by these compounds might explain, at least in part, the previously unexplained difference between concentrations of LAs required for their anti-inflammatory action in vitro and in vivo. This study suggests a target site for LAs within a Gq-coupled signaling pathway.

LOCAL anesthetics (LAs) have a variety of actions in addition to sodium channel blockade. Of particular interest are reports indicating that these compounds modulate the inflammatory response. In vitro they prevent or reduce inflammatory disorders such as reperfusion injury in heart, lung, and brain, as well as endotoxin- or hypoxia-induced pulmonary injury. In vitro, LAs inhibit signaling actions of macrophages and granulocytes, which mediate early steps of the inflammatory response (see Hollmann et al.1 for review).

Unfortunately, the mechanisms behind these potentially beneficial effects of LAs are largely unknown. It is clear, however, that these actions do not result primarily from sodium channel blockade. The anti-inflammatory effects of LAs observed in vitro cannot easily explain those found in the clinical setting, since concentrations required to achieve inhibition of inflammatory cells in vitro are approximately three orders of magnitude greater than plasma concentrations obtained after intravenous or epidural administration of LAs.2 LAs inhibit signaling through several G protein- coupled receptors. Because many inflammatory mediators signal through such receptors, LAs may modulate inflammatory responses by inhibiting inflammatory mediator signaling. We recently showed that clinically relevant concentrations of LAs inhibit several actions of the phospholipid mediator lysophosphatidate on human polymorphonuclear neutrophils (hPMNs).3,4

Human polymorphonuclear neutrophils are of great importance in host defense, as they move actively to the site of inflammation (chemotaxis), where a multicomponent enzyme complex, nicotinamide adenine dinucleotide phosphate oxidase, generates toxic oxygen metabolites (O2•−, H2O2, HOCl, and OH•). hPMNs exist in one of three states: quiescent, primed, or active. Priming refers to a process whereby the response of hPMNs to a subsequent activating stimulus is potentiated. Release of oxygen metabolites is markedly enhanced when hPMNs have previously been primed.5 The priming process has been shown to be a critical component of hPMN-mediated tissue injury both in vitro and in vivo.5

In this study we investigated the effects of LAs on hPMN priming by platelet-activating factor (PAF), a representative inflammatory mediator. PAF is an established mediator in early acute respiratory distress syndrome,6 a typical postoperative inflammatory disorder. It plays a pivotal role in lipopolysaccharide-induced lung injury,7–10 and alveolar PAF levels are increased in acute respiratory distress syndrome.11 PAF inhibition reduces endotoxin-induced lung dysfunction12 and pulmonary injury after cardiopulmonary bypass.13

Our findings indicate that LAs inhibit PAF-mediated priming. The priming pathway is phospholipase C (PLC)- and protein kinase C (PKC)-dependent, and the main site of LA action is upstream from PKC.
of hPMNs in the suspension, which was then diluted.

The reduction of cytochrome c (cyt c) is catalyzed by superoxide dismutase (SOD) when SOD is inhibited by another radical oxygen metabolite, the selective contribution of cyt c can be determined. Catalase was used to degrade H$_2$O$_2$ into H$_2$O and O$_2$, preventing H$_2$O$_2$ from reoxidizing the reduced cytochrome c. Such H$_2$O$_2$-dependent oxidation of reduced cytochrome c would give inaccurate, falsely low results.

The reaction was activated by either adding N-formylmethionine-leucyl-phenylalanine (fMLP) or phosphor myristate acetate (PMA), and the change in absorbance at 550 nm was monitored over time. The reference sample was measured immediately after, and O$_2$-dependent cytochrome-c reduction was determined by subtracting the reference value from the study sample value. To test LA effects on either priming or activation, hPMNs were incubated for 60 min at 37°C in various concentrations of different LAs before hPMN activation with fMLP. When PAF was used as the priming agent, hPMNs were incubated for 5 min before activation with fMLP or PMA, since priming effect by PAF has been shown to be maximal after 5 min.

Using fMLP for activation, O$_2$ generation at time points 0, 5, 10, 14, 16, and 18 min was measured, because we determined in a previous study maximal activation after 16 min. PMA-induced superoxide anion production was determined at time points 0 to 50 min every 5 min. O$_2$ production was calculated using as conversion factor 47.4 μmol (1/21.1 μM$^{-1}$ difference of extinction coefficient between oxidized and reduced cytochrome c at 550 nm) O$_2$ per unit change in absorbance.

We measured extracellular (rather than total) PMN-mediated release of O$_2$ because it is the release of oxygen metabolites into the extracellular milieu that may directly damage cells in the surrounding microenvironment. Extracellular O$_2$ release can be monitored by either end-point or kinetic assays. In spectrophotometric end-point assays, the total amount of O$_2$ that is released into the extracellular environment by a fixed number of PMNs is quantitated after a given incubation time. A kinetic assay determines both the total amount of O$_2$ produced and the rate of O$_2$ release over time. We used a kinetic assay because the kinetics of PMN O$_2$ release are nonlinear and vary with different priming and activating stimuli.

**Reagents**

Hank’s balanced salt solution (without phenol red, with Ca$^{2+}$–Mg$^{2+}$) was obtained from Whittaker Bioproducts.
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Platelet-activating Factor Priming Is Mediated by a Pertussis Toxin–Insensitive G Protein

We studied the intracellular signaling pathways involved in PAF-induced priming. Because both PAF and fMLP act through G protein–coupled receptors, we determined the effects of PTX on PAF-primed–fMLP-activated O2 production. Preincubation with PTX (0.5 μg/ml) for 90 min caused a modest but significant (P = 0.001, t test) reduction in superoxide anion production to 73.4 ± 9.6% of control response (13.4 ± 3.1 nmol/106 cells; fig. 2C). This suggests that Gq and/or G11 proteins are involved but are not the only G-protein subtypes mediating O2 production.

We also investigated the effect of PTX on O2 production of hPMNs primed with PAF (10–6 m) and activated with the PKC agonist PMA, bypassing the G-protein step of the activation pathway. As illustrated in figure 2D, O2 production in response to 1 nM PMA (4.7 ± 1.1 nmol/106 cells) increased significantly after incubation with PAF for 5 min (14.4 ± 2.8 nmol/106 cells). In contrast to our findings using fMLP as activator, we observed no significant effect of PTX on PAF-primed–PMA-activated O2 production (PTX, 14.1 ± 0.4 nmol/106 cells; control, 13.9 ± 0.4 nmol/106 cells, n = 19; P = 0.105, t test; fig. 2E). Based on these experiments, we conclude that activation is partially PTX-sensitive, but only PTX-insensitive G proteins are required for the priming process.

U-73122 Abolishes Platelet-activating Factor Priming of Phorbol Ester-Induced Superoxide Anion Production

Because we determined that PAF priming is mediated through PTX-insensitive G proteins (presumably Gq/11), we studied if priming depends on activation of PLC, the main effector activated by these G proteins.

We primed hPMNs with PAF and measured superoxide anion production after agonist stimulation in the absence and presence of the putative PLC antagonist 1-(6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-1H-pyrrole-2,5-dione (U-73122). Because the activation pathway may also partly involve PLC,15 we used PMA as the activating stimulus. The priming effect of PAF was abolished completely (5.0 ± 1.9 nmol/106 cells) when hPMNs were preincubated for 60 min in U-73122 (10–6 m; fig. 2F), indicating that PLC mediates PAF priming.

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Protein Kinase C Inhibitors Abolish the Priming Effect of Platelet-activating Factor

Diacetylglycerol, generated by the action of PLC on phosphatidylinositolbisphosphate, is the endogenous activator of PKC. Because PAF priming is mediated through a PLC mechanism, we hypothesized that PKC activation plays a role in this pathway. We therefore investigated whether PKC inhibitors block PAF priming. Sixty-minute pretreatment of hPMNs with one of two different PKC inhibitors completely abolished the priming effect by PAF and reduced response sizes to those obtained with FMLP alone (9.5 ± 4.8% of primed response; fig. 3A). Bisindolylmaleimide (10⁻⁶ M) reduced superoxide anion production to 12.9 ± 3.6% of control response, and chelerythrine (10⁻⁵ M) inhibited O₂⁻ production to 5.9 ± 3.1% of control response. No significant difference (P > 0.05, one-way analysis of variance, Dunnett correction) between FMLP-induced responses and those elicited after priming-activation in the presence of bisindolylmaleimide or chelerythrine was obtained. This finding suggests that, in contrast to the activation process, priming through PAF is critically dependent on PKC.

Phorbol Ester Both Activates and Primes

If priming is dependent on PKC, one should be able to induce priming by direct PKC activation. At the same time, PMA is able to induce superoxide anion production (although it has been shown that PKC is not required for activation of hPMNs¹⁴). Therefore, it seems likely that PMA treatment of hPMNs would both prime and activate the cells. To determine the maximal priming-activating dose of PMA, we performed a concentration-response study. After adding PMA to hPMNs, superoxide anion production was measured for 40 min (because pilot experiments determined this as sufficient time for O₂⁻ production to plateau after PMA stimulation; data not shown). As indicated in figure 3B, maximal superoxide anion production was in-
duced by 100 nM PMA (23.6 ± 4.5 nmol/10⁶ cells). Using this PMA concentration to provide maximal stimulation, we next tested our hypothesis that PMA treatment of hPMNs would both prime and activate the cells. We determined first the time course of O₂² production after stimulation with PMA (100 nM) and compared it with that obtained after PAF-MLP treatment. As shown in figure 3C, PMA induced a slower respiratory burst than did PAF-MLP. Thirty minutes after PMA administration, superoxide anion production reached the maximal level. At this time point, measured O₂² production was 174.6 ± 16.6% of the response (13.6 ± 1.6 nmol/10⁶ cells) obtained after stimulation with fully priming and activating concentrations of PAF and fMLP. The slow time course suggests that priming may be taking place at the same time as activation.

If PMA indeed acts as a full priming agent, PAF priming of PMA responses might accelerate the onset of priming but should not increase the maximal response. Therefore, we tested the ability of PAF (10⁻⁶ M) to prime PMA (100 nM)-induced O₂² production. Although PAF accelerated the time course of superoxide production by PMA (fig. 2D), after 30 min no significant difference between O₂² production induced by PMA with or without PAF pretreatment was present (fig. 3C). These findings suggest that after 30 min, superoxide anion production by PMA results from a combined priming–activating effect, most likely because of a dual action of PKC on the priming and activation pathways.

Lidocaine and Tetracaine Inhibit Human Polymorphonuclear Neutrophil Priming

After mapping the PAF priming pathway, we studied the effects of LA on priming. We confirmed the findings from our previous study (Durieux ME, unpublished observation, 2000), showing that LAs (lidocaine, tetracaine, and the permanent charged lidocaine analog QX314) do not affect the activation process of hPMNs (data not shown). We then investigated the effect of the most commonly used amide LA, lidocaine (fig. 4A), and the ester LA tetracaine (fig. 4B) on PMA priming by PAF. hPMNs were incubated for 60 min in various concentrations (10⁻⁴ M to 10⁻⁶ M) of the LAs, before priming with PAF. Both LAs inhibited superoxide anion production in a modestly concentration-dependent manner over the range tested. Even at an LA concentration (10⁻⁶ M) commonly attained in plasma after epidural or intravenous administration,18–21 O₂² production was attenuated significantly (to 73.5 ± 6.7% of control response for lidocaine and 68 ± 12.1% of control response for tetracaine; P < 0.05, one-way repeated-measurement analysis of variance, Dunnett correction) as compared with the PAF (10⁻⁶ M)-primed–fMLP (10⁻⁶ M)-activated control response (11.8 ± 2.5 nmol/10⁶ cells for lidocaine and 14.2 ± 7.3 nmol/10⁶ cells for tetracaine experiments). At the highest concentration tested (10⁻⁴ M), tetracaine (42.8 ± 13% of control response) was found to inhibit O₂² production more effectively (P = 0.001, unpaired t test) than lidocaine (62.8 ± 5.1% of control response).

Ropivacaine and Bupivacaine Are Less Potent Inhibitors of Human Polymorphonuclear Neutrophil Priming

Because the amide LA ropivacaine has received significant interest as an inflammatory modulator and has

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undergone clinical studies of its use in inflammatory bowel disease,22 we determined its action on PAF (10^{-6} M)-primed–fMLP (10^{-6} M)-activated hPMNs. Both stereoisomers of ropivacaine (10^{-4} M) inhibited PAF priming with a modest but significant (P < 0.05, one-way repeated-measurement analysis of variance, Student-Newman-Keuls correction) difference between S(-)-ropivacaine (78.8 ± 3.6% of control response) and the R(+) -isomer (67 ± 5.1% of control response; fig. 4C). In addition, we tested the inhibitory potency of bupivacaine, which is structurally similar to ropivacaine but is much more lipophilic. At a concentration of 10^{-4} M, bupivacaine showed a weak (81.4 ± 4.3% of control response) but nonetheless significant (P < 0.05, paired t test) inhibition of superoxide anion production compared with PAF (10^{-6} M)-primed–fMLP (10^{-6} M)-activated control cells (13.2 ± 4.2 nmol/10^6 cells; fig. 4C). Ropivacaine and bupivacaine were similar in their inhibitory effect (P = 0.06, t test [n = 18]). Both compounds showed a lower inhibitory potency on priming than did lidocaine or tetracaine, and their effect appears not to depend on lipophilicity.

### Priming of Human Polymorphonuclear Neutrophils by Platelet-activating Factor or Lysophosphatidate Is Inhibited More Profoundly by Uncharged Local Anesthetics

To obtain additional information about the site of LA action on PAF-induced priming, we tested the effects of the permanently charged and therefore membrane-impermeant lidocaine analog QX314, and the permanently uncharged membrane permeant LA benzocaine. We compared both with the effect of lidocaine on PAF (10^{-6} M)-primed–fMLP (10^{-6} M)-activated hPMNs (fig. 4D). Benzocaine (10^{-4} M) inhibited priming profoundly (to 33.2 ± 4.5% of control response), whereas QX314 (10^{-4} M) had a modest effect (86.4 ± 6.7% of control response). Lidocaine, as a partially charged compound,
was intermediate in effect and inhibited to 59.3 ± 5.9% of control response.

For comparative purposes, we also determined the effects of QX314 and benzocaine on lysophosphatidate (10⁻⁴ M) priming in neutrophils (fig. 4D). The findings were very similar to those obtained using PAF. Benzocaine (10⁻⁴ M) inhibited primed responses to 39.6 ± 4.8% of control response, whereas QX314 was without effect (101 ± 13.6% of control response). The latter finding is in agreement with our previous observation showing that lysophosphatidate signaling in Xenopus oocytes is not affected by extracellularly applied QX314.²³ Lidocaine inhibited to 69 ± 5.6% of control.

These findings suggest that lysophosphatidate and PAF signaling in hPMNs are affected similarly by LAs. There appears to be, at best, a limited role for extracellular block by charged compounds. The major site of action, therefore, is either an uncharged extracellular domain or an intracellular site, which may be charged or uncharged.

The Site of Action of Lidocaine Is Located Upstream of Protein Kinase C and Does Not Involve Gᵢₒ

To determine the site of action of lidocaine (10⁻⁴ M) in more detail, we studied its effect on hPMNs primed and activated by PMA. We studied the effects 30 min after PMA application, at which time both priming and activation are complete (fig. 3B). As shown in figure 5A, lidocaine was without effect when cells were stimulated with 100 nM PMA (lidocaine, 21.7 ± 0.5 nmol/10⁶ cells; control, 22 ± 0.8 nmol/10⁶ cells; n = 17; P = 0.2, t test) or 10 nM PMA (lidocaine, 13.1 ± 0.4 nmol/10⁶ cells; control, 13.3 ± 0.4 nmol/10⁶ cells; n = 20; P = 0.122, t test). Thus, lidocaine did not affect PMA-induced O₂⁻ production, suggesting that neither in activation nor priming pathway is its target site located downstream of PKC.

Finally, we determined if inhibition of Gᵢᵢₒ-Gᵢₒ may play a role in the effects of lidocaine. We preincubated hPMNs in PTX (0.3 μg/ml) and lidocaine (10⁻⁴ M). As demonstrated in figure 5B, superoxide anion production was reduced to 31.4 ± 7.9% of control response. Lidocaine still exerted an approximately 40% inhibition of PAF (10⁻⁶ M)-primed-fMLP (10⁻⁶ M)-activated hPMNs in addition to the 31% inhibition by PTX. This is similar to its effect in the absence of PTX (fig. 4A). Taking this finding together with the previously described lack of lidocaine inhibition on fMLP-induced activation, the main target site for lidocaine is likely to be different from Gᵢᵢₒ.

Discussion

In the current study, we have shown that PAF primes neutrophils through a pathway dependent on PTX-insensitive G proteins, PLC, and PKC. PKC activation is both necessary and sufficient for this process. In addition, we show that clinically relevant concentrations of LAs selectively inhibit priming but not fMLP-induced activation. Estrogen LA exerted the most profound inhibitory effect, whereas inhibitory potency of amide LA increased with increased uncharged fraction. The main target site for LAs in the PAF priming pathway is located upstream of PKC.

The concentrations at which these effects take place are much less than those required to block sodium channels. Lidocaine showed significant inhibitory effects on priming, even at concentrations commonly obtained in plasma of patients after epidural or intravenous administration (approximately 0.5–5 μg/ml, corresponding to 2–20 μM)²⁸; for example, intravenous administration of lidocaine at 2–4 mg/min leads to plasma concentrations of 1–3 μg/ml (4–12 μM) after 150 min.¹⁹ A 2-mg/kg intravenous bolus dose of lidocaine results in peak plasma levels of 1.5–1.9 μg/ml (6–8 μM) after 15 min.²⁰ Similar plasma concentrations are obtained after epidural administration.²¹ The effects of LAs on priming of

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hPMNs might be one explanation for the discrepancy between concentrations required to achieve antiinflammatory effects of LA in vitro and in vivo. Virtually all in vitro studies of LA effects on hPMNs investigated activation. To our knowledge, the effects of LAs on priming have not been reported previously, although this state of hPMNs should be more representative for in vivo inflammatory processes. Primed hPMNs have been identified in the peripheral blood of patients after blunt trauma, acute respiratory distress syndrome, and bacterial or fungal infections. hPMN priming has been shown to be critical for the induction of endothelial injury and lung damage in vivo. Because LAs, in reasonable concentrations, did not block unprimed neutrophil metabolic activity, this might explain why antiinflammatory LA concentrations required to block activation in vitro are generally much greater than those required to block inflammatory responses in vivo. Another factor may be that duration of LA incubation (60 min) is longer in our study than in most other studies. We believe that this mimics the clinical setting more closely.

We used different LAs to determine which chemical properties might enhance or reduce their inhibitory potency. Because tetracaine and benzocaine (ester LA) showed inhibition comparable to or even more profound than that obtained with amide LA, the inhibitory effect appears independent of the linkage type within the LA molecule, consistent with our findings on lyso-phosphatidate-primed hPMNs (Durieux ME, unpublished observation, 2000). For the amide LA lidocaine (ropivacaine, bupivacaine, and QX314), we observed an increase in inhibitory potency with increased uncharged fraction. Lidocaine, a representative mostly uncharged amide LA at physiologic pH, exerted the most profound inhibition within the group of amide compounds, whereas bupivacaine (highly lipophilic and highly charged at pH 7.4) and QX314 (permanently charged) showed the least inhibitory effect. Obviously, these findings may reflect a need for the compound to traverse the cell membrane to reach its site of action. Ropivacaine, which is structurally similar to bupivacaine but similar in lipophilicity to lidocaine, showed no significant difference in inhibitory potency as compared with bupivacaine, suggesting that the inhibitory effect is independent of lipophilicity of the compound.

The site of local anesthetic action is of obvious interest. Interaction with sodium channels is unlikely, as these are not present in hPMNs, and concentrations used are less than those required to block sodium channels. Lidocaine failed to inhibit PMA-induced superoxide anion production, indicating that the major site of action of lidocaine has to be located upstream of PKC in the PAF priming pathway. The effects of LAs on the activation process were excluded in this and also in our previous study (Durieux ME, unpublished observation, 2000). The site of action is therefore likely to be located between receptor and PKC. In Xenopus oocytes, we previously showed that LAs do not interfere with the PLC–inositol trisphosphate–calcium pathway. If these findings can be extrapolated to hPMNs, the PAF receptor or G protein(s) itself seem to be the most likely target. QX314 was virtually without effect when applied extracellularly, excluding a charged extracellular site as the main target. However, an uncharged extracellular site
cannot be ruled out. In oocytes, we have shown intracellular QX314 to inhibit Gq proteins selectively.30 Because we determined that PAF priming is mediated through a PTX-insensitive G protein, we hypothesize that Gq is a likely LA target in inhibiting PAF priming. This is in agreement with the finding that lidocaine still exerted its inhibitory action in the presence of PTX and with previous findings from our group showing that neither Gs (Durieux ME, unpublished observation, 2000) nor Gq signaling is inhibited by relevant LA concentrations. Although Gq-mediated signaling is PTX-insensitive, it is unlikely to be involved because increases in cyclic adenosine monophosphate levels, resulting from Gs activation, inhibit neutrophil functions.31-33

Protein kinase C activation with PMA is able to both prime and activate hPMNs. PMA induces respiratory burst in a concentration-dependent manner. We determined that PMA-induced superoxide anion production could be primed effectively with PAF after 15 min. However, maximal PMA-induced O2 production obtained after 30 min is most likely the result of a combined priming and activation effect, as PAF is no longer able to increase O2 production further. Lack of priming effect on PMA-stimulated superoxide anion production by PAF after 30 min cannot be attributed to loss of PAF action, because its priming effect on fMLP-induced O2 production did not change over a 50-min time course.

Using different PKC inhibitors (including the broad-range PKC inhibitor bisindolylmaleimide), we demonstrated that PAF priming is inhibited completely by PKC blockade, indicating that the priming process is critically dependent on PKC. This is in contrast to activation by fMLP. Pongracz et al.14 showed that several PKC inhibitors were not able to affect fMLP-induced superoxide anion production.

Persitux toxin partially inhibited superoxide anion production in PAF-primed, fMLP-activated hPMNs but not in PAF-primed, PMA-activated cells. hPMNs were incubated in PTX for 90 min before their activation, as reported by Christiansen.15 We used a PTX concentration of 0.3 μg/ml when studying fMLP activation, as this concentration has been shown to inhibit fMLP-induced superoxide anion production by approximately 50%.15 Higher concentrations would have abolished fMLP activation completely and were therefore used only when studying PAF-primed-PMA-activated cells. Despite the greater concentration, PTX was without effect on PAF-primed, PMA-activated hPMNs. The most likely explanation is that PTX partially inhibited fMLP-induced activation but was without effect on priming. In fact, several investigators reported that fMLP-induced signaling in hPMNs is mediated by PTX-sensitive G proteins.34-36

Our hypothesis of the signaling pathways involved in superoxide anion production of PAF-primed and fMLP-activated hPMNs is illustrated in figure 6.

In conclusion, our data provide new insights into the mechanism of hPMN priming and, in addition, suggest a mechanism by which LAs may exert some of their anti-inflammatory actions.

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