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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 G_q-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 antiinflammatory action *in vitro* and *in vivo*. This study suggests a target site for LAs within a G_q-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 vivo* 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 (O_2^-, H_2O_2, 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 dysfunction[12] 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.

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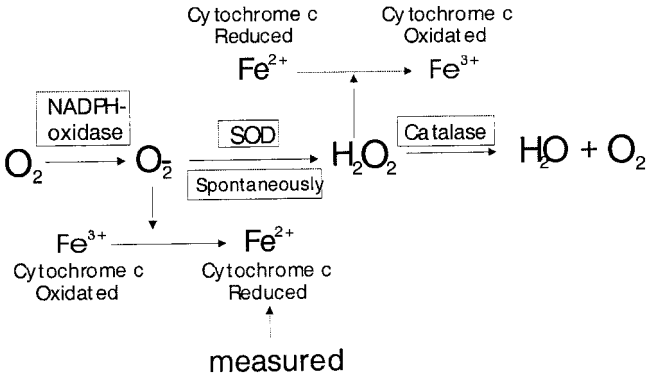

Fig. 1. Schematic overview of the production and conversion of O_2^- and the corresponding oxidation and reduction of cytochrome *c*.

Materials and Methods

The study protocol was approved by the University of Virginia Institutional Review Board.

Preparation of Human Polymorphonuclear Neutrophils

Human venous blood was obtained from healthy donors who had not used any medication for at least 2 weeks. Blood was heparinized (10 U/ml), and hPMNs were isolated by a one-step separation procedure. After 40 min of centrifugation at 500*g*, hPMNs were washed three times with Hank's balanced salt solution (HBSS; containing 10 U/ml heparin) and centrifuged after each washing step at 350*g* (20 min after first wash, 10 min after second and third wash). hPMNs were then resuspended in 5 ml HBSS and counted using a hemocytometer. Unopette *in vitro* Diagnostic System (Becton Dickinson, Franklin Lakes, NJ) was used for the enumeration of hPMNs in the suspension, which was then diluted with HBSS to obtain a final neutrophil suspension of 5×10^6 cells/ml. Purity of our hPMN suspension, assessed by morphology, exceeded 98%. The viability of neutrophils was checked by Trypan blue exclusion and was always found to be greater than 94%. All preparation and assays were performed at room temperature.

Superoxide Anion (O_2^-) Generation

Cytochrome-*c* Reduction Assay. We used the cytochrome-*c* reduction assay to measure extracellular O_2^- production by activated hPMNs as described previously.[14,15] O_2^- generation was measured spectrophotometrically as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* (fig. 1).

Superoxide anion production was measured over time by the absorbance of cytochrome *c* at 550 nm. The reaction was performed in a spectrophotometer (Genesys 5, Spectronic Instruments, Rochester, NY). The reaction mixture was prepared by placing 700 μl buffer (HBSS + bovine serum albumin 0.1%), 200 μl of hPMN suspension (final concentration, 10^6 cells/ml), and 100 μl cytochrome *c* (from horse heart, 3.7 mg/ml) with catalase (0.14 mg/ml) in a 1-ml cuvette. The reference sample was prepared the same way, but in addition, 10 μl SOD (10^{-2} M) was added to the mixture. Many electron donors in addition to O_2^- can reduce cytochrome *c*, but only O_2^- is destroyed by SOD, by catalyzing its conversion to H_2O_2. By subtracting the absorbance of the SOD reaction (representing cytochrome-*c* reduction caused by other radical oxygen metabolites), the selective contribution of O_2^- can be determined. Catalase was used to degrade H_2O_2 into H_2O and O_2, preventing H_2O_2 from reoxidizing the reduced cytochrome *c*. Such H_2O_2-dependent oxidation of reduced cytochrome *c* would give inaccurate, falsely low results.

The reaction was activated by either adding *N*-formyl-methionine-leucyl-phenylalanine (fMLP) or phorbol 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.[16] 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.[3,4] 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 mM^{-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 Bioprod-

ucts (Walkersville, MD), and SOD (from bovine liver), fMLP, cytochrome *c* (from horse heart), catalase (from bovine liver), PMA, U73122, bisindolylmaleimide, chelerythrine, and pertussis toxin (PTX) were obtained from Sigma Chemicals Co. (St. Louis, MO). Ficoll-Hypaque and bovine serum albumin (protease free bovine albumin fraction–fatty acid free) were obtained from ICN Biomedicals, Inc. (Aurora, OH). Polymorph (Westbury, NY) neutrophil isolation medium was obtained from Cardinal Associates (Santa Fe, NM). PAF (1-alkyl-2-acetoyl-*sn*-glycero-3-phosphocholine) was obtained from Avanti polar lipids (Alabaster, AL). Lidocaine and QX 314 were gifts from AstraZeneca Pharmaceuticals LP (Westborough, MA).

Statistical Analysis

Data are reported as mean ± SD. Leukocyte metabolic activity is reported either as superoxide anion production or as percentage change from control. Blood from at least eight donors was used for each data point. Groups were compared using either paired Student *t* test or one-way repeated-measurement analysis of variance, followed by Dunnett or Student-Newman-Keuls correction (as described in Results), if necessary. $P < 0.05$ was considered significant. SigmaStat 2.0 (Jandel Scientific Corporation, San Rafael, CA) was used for all statistical analyses.

Results

Because hPMNs were to be incubated in LA for significant duration before the activation assay, we determined, in pilot experiments, the effect of incubation in buffer (37°C, 0–60 min) as well as the effect of movement (by a shaking water bath) on O_2^- production. Neither affected O_2^- production as compared with untreated control hPMNs (data not shown). We also determined any interference of PAF with the cytochrome *c* assay. PAF did not have any significant effect on absorbance in a neutrophil-free solution as compared with control (data not shown).

Platelet-activating Factor Primes but Does Not Activate Human Polymorphonuclear Neutrophils

After these control experiments, we determined the ability of PAF to activate and/or prime hPMNs. hPMNs were added to the assay solution, and baseline activity was measured to exclude activating effects of the isolation process itself. Activator was then added to the samples, and absorbance was measured for the next 20 min. PAF in concentrations between 10^{-4} and 10^{-8} M induced virtually no activation. In contrast, fMLP (10^{-6} M, a concentration selected as it gave optimal responses in our previous studies[3,4]) induced superoxide anion production of approximately 1.9 ± 0.6 nmol/10^6 cells, confirming the functionality of the assay (fig. 2A). When pretreated for 5 min with various PAF concentrations (10^{-5} M to 10^{-8} M), responses to fMLP were increased significantly (fig. 2B), indicating that hPMNs could be primed appropriately by PAF, as described previously.[16,17] Maximal priming effect (11.1 ± 3.9 nmol/10^6 cells, an almost sixfold increase) was obtained with 10^{-6} M PAF. Therefore, this concentration was used for the remainder of the study.

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 O_2^- production. Preincubation with PTX (0.3 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/10^6 cells; fig. 2C). This suggests that G_i and/or G_o proteins are involved but are not the only G-protein subtypes mediating O_2^- production.

We also investigated the effect of PTX on O_2^- 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, O_2^- production in response to 1 nM PMA (4.7 ± 1.1 nmol/10^6 cells) increased significantly after incubation with PAF for 5 min (14.4 ± 2.8 nmol/10^6 cells). In contrast to our findings using fMLP as activator, we observed no significant effect of PTX on PAF-primed–PMA-activated O_2^- production (PTX, 14.1 ± 0.4 nmol/10^6 cells; control, 13.9 ± 0.4 nmol/10^6 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 $G_{q/11}$), 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/10^6 cells) when hPMNs were preincubated for 60 min in U-73122 (10^{-6} M; fig. 2F), indicating that PLC mediates PAF priming.

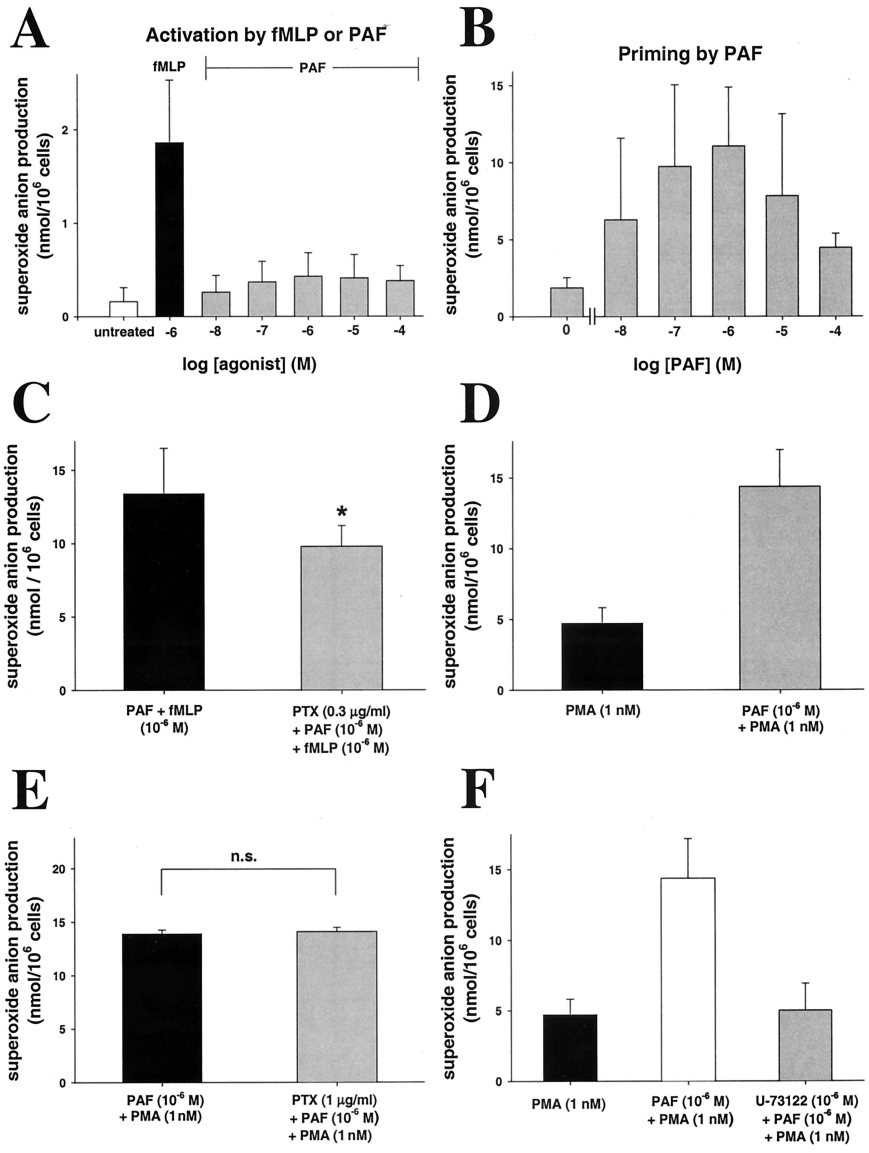

Fig. 2. (*A*) N-formylmethionine-leucyl-phenylalanine (fMLP)- and platelet-activating factor (PAF)-induced human polymorphonuclear neutrophil (hPMN) activation. fMLP (black bar) induces superoxide anion production, whereas PAF has virtually no activating properties (gray bars). Untreated: baseline superoxide anion production by untreated hPMNs (white bar). (*B*) Superoxide anion production by hPMNs activated by fMLP (left bar, data from fig. 2A) or after priming for 5 min with PAF. (*C*) Preincubation of hPMNs with petussis toxin (PTX; grey bar) inhibits superoxide anion production of PAF-primed–fMLP-activated hPMNs. *Significant difference compared with PAF-primed–fMLP-activated control response. (*D*) PAF increases superoxide anion production in hPMNs induced by 1 nM phorbol myristate acetate (PMA). (*E*) PTX (1 μg/ml) has no significant effect on PAF-primed–PMA-activated superoxide anion production (gray bar) compared with PAF-primed–PMA-activated control hPMNs (black bar). (*F*) PAF increases superoxide anion production in hPMNs induced by 1 nM PMA (data from fig. 2D). U-73122, a phospholipase C antagonist, completely abolishes the priming effect of PAF (gray bar). See text for details.

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

Diacylglycerol, 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 O_2^- 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,[14] 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 hPMNs[14]). 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 O_2^- production to plateau after PMA stimulation; data not shown). As indicated in figure 3B, maximal superoxide anion production was in-

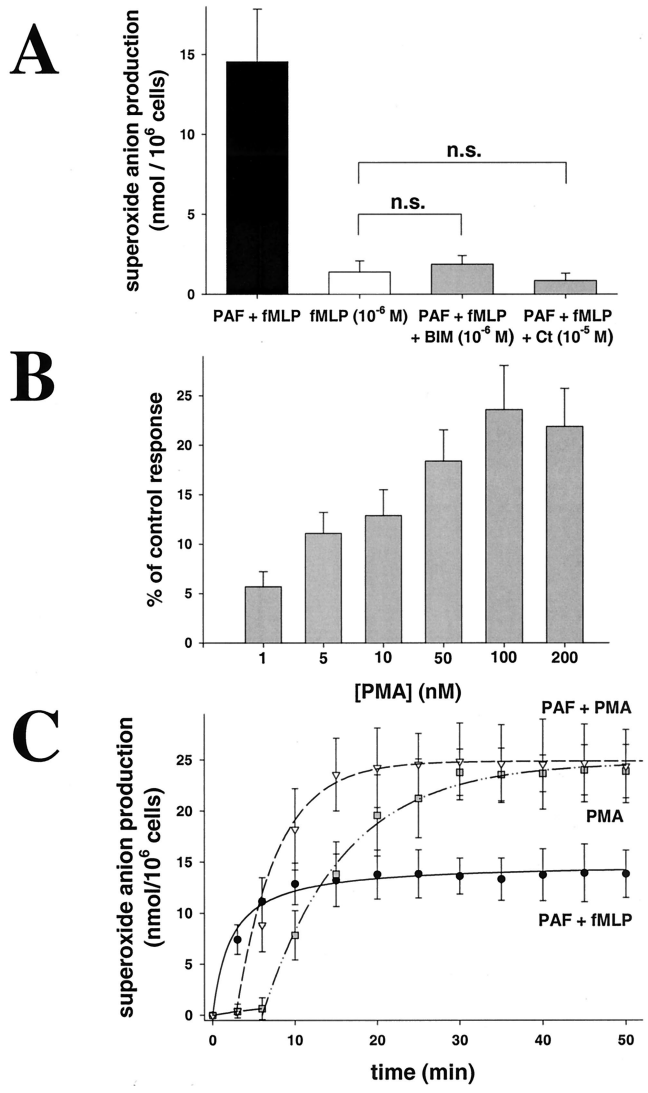

Fig. 3. (*A*) Platelet-activating factor (PAF)-primed–N-formylmethionine-leucyl-phenylalanine (fMLP)-activated O_2^- production by hPMNs (black bar) is inhibited after 60-min pretreatment with bisindolylmaleimide (BIM; gray bar) or chelerythrine (Ct; gray bar) to a level not significantly different from that obtained with fMLP activation alone (white bar). (*B*) Concentration–response relation for superoxide anion production induced by various concentrations of phorbol myristate acetate (PMA; 1–200 nM) measured 40 min after adding PMA to human polymorphonuclear neutrophils (hPMNs). (*C*) Time course of superoxide anion production induced by either fMLP activation and previous incubation with PAF for 5 min, PMA alone, or PMA and 5-min preincubation with PAF. See text for details. n.s. = not significant.

duced by 100 nM PMA (23.6 ± 4.5 nmol/10^6 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 O_2^- production after stimulation with PMA (100 nM) and compared it with that obtained after PAF–fMLP treatment. As shown in figure 3C, PMA induced a slower respiratory burst than did PAF–fMLP. Thirty minutes after PMA administration, superoxide anion production reached the maximal level. At this time point, measured O_2^- production was 174.6 ± 16.6% of the response (13.6 ± 1.6 nmol/10^6 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 O_2^- production. Although PAF accelerated the time course of superoxide production by PMA (fig. 2D), after 30 min no significant difference between O_2^- production induced by PMA with or without PAF pretreatment was present (fig. 3C). These finding suggests 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 hPMN 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] O_2^- 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/10^6 cells for lidocaine and 14.2 ± 7.3 nmol/10^6 cells for tetracaine experiments). At the highest concentration tested (10^{-4} M), tetracaine (42.8 ± 13% of control response) was found to inhibit O_2^- 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

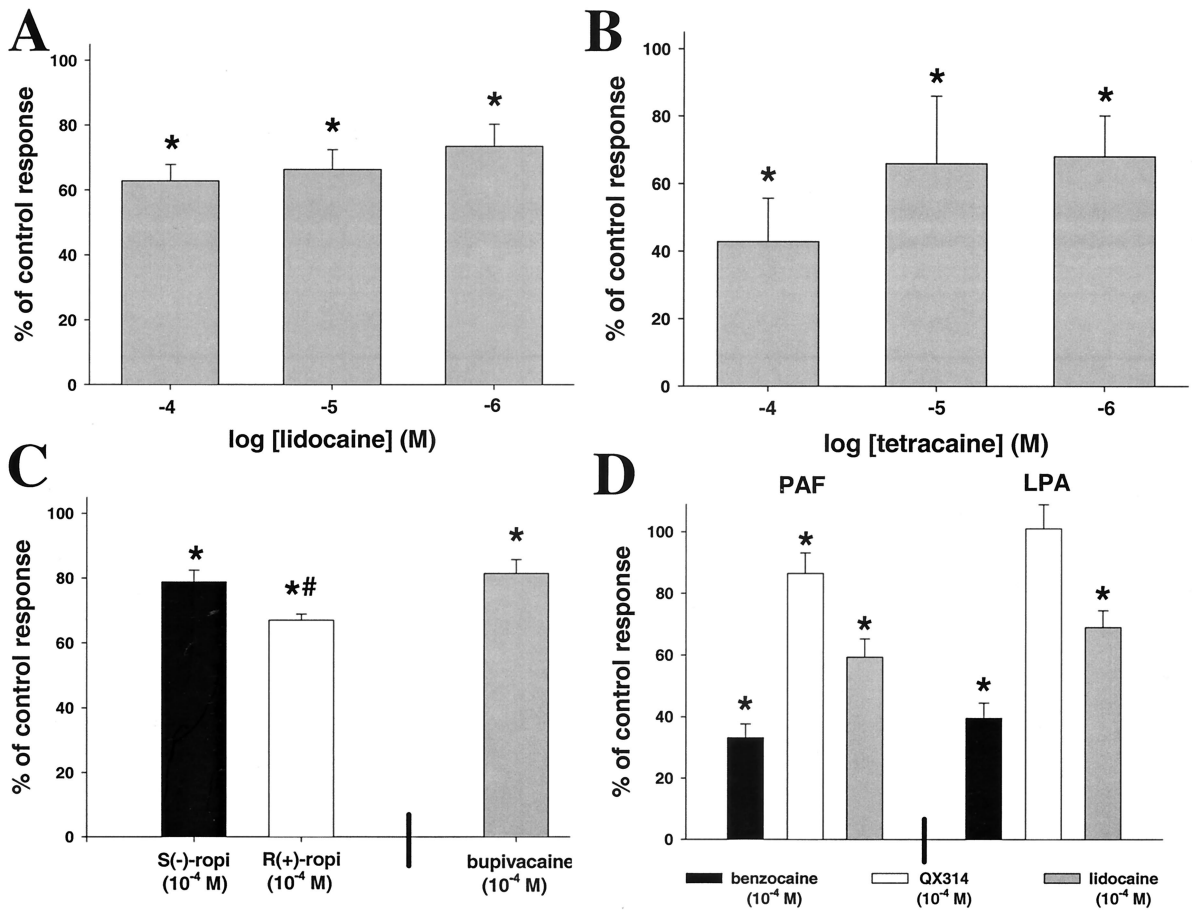

Fig. 4. (*A* and *B*) Concentration-dependent inhibition of superoxide anion production in human polymorphonuclear neutrophils (hPMNs) after 60-min incubation in lidocaine (*A*) or tetracaine (*B*) before priming with platelet-activating factor (PAF) and activation with N-formylmethionine-leucyl-phenylalanine (fMLP). (*C*) Effects of 60-min preincubation with ropivacaine isomers or bupivacaine on PAF-primed–fMLP-activated hPMNs. #Significant difference between ropivacaine stereoisomers. *Significant difference from PAF-primed–fMLP-activatred control. (*D*) Effects of 60-min preincubation of hPMNs with QX314, benzocaine, or lidocaine on lysophosphatidate (LPA) or PAF-primed–fMLP-activated hPMNs. *Significant difference compared with with PAF-primed and fMLP-activated control response; black dividers on the x-axis indicate different experiments with different control responses. See text for details.

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 $G_{i/o}$

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/10^6 cells; control, 22 ± 0.8 nmol/10^6 cells; n = 17; $P = 0.2$, *t* test) or 10 nM PMA (lidocaine, 13.1 ± 0.4 nmol/10^6 cells; control, 13.3 ± 0.4 nmol/10^6 cells; n = 20; $P = 0.122$, *t* test). Thus, lidocaine did not affect PMA-induced O_2^- production, suggesting that neither in activation nor priming pathway is its target site located downstream of PKC.

Finally, we determined if inhibition of G_i–G_o 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 $G_{i/o}$.

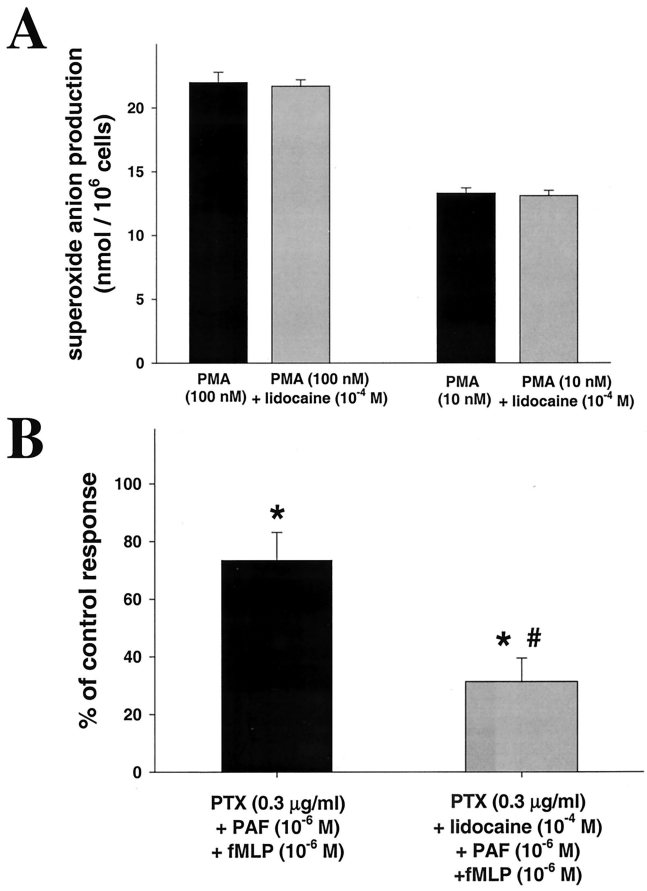

Fig. 5. (*A*) Lidocaine, when incubated for 60 min before human polymorphonuclear neutrophil (hPMN) activation with phorbol myristate acetate (PMA), does not affect superoxide anion production (after 30 min) significantly. (*B*) Preincubation (90 min) of hPMNs with pertussis toxin (PTX; 0.3 μg/ml) inhibits superoxide anion production only partially, whereas combined pretreatment with lidocaine (10^{-4} M) and PTX (0.3 μg/ml, gray bar) reduces O_2^- production to 31.4 ± 7.9% of control response. PAF = platelet-activating factor; fMLP = *N*-formylmethionine-leucyl-phenylalanine.

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. Ester 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)[18]; 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

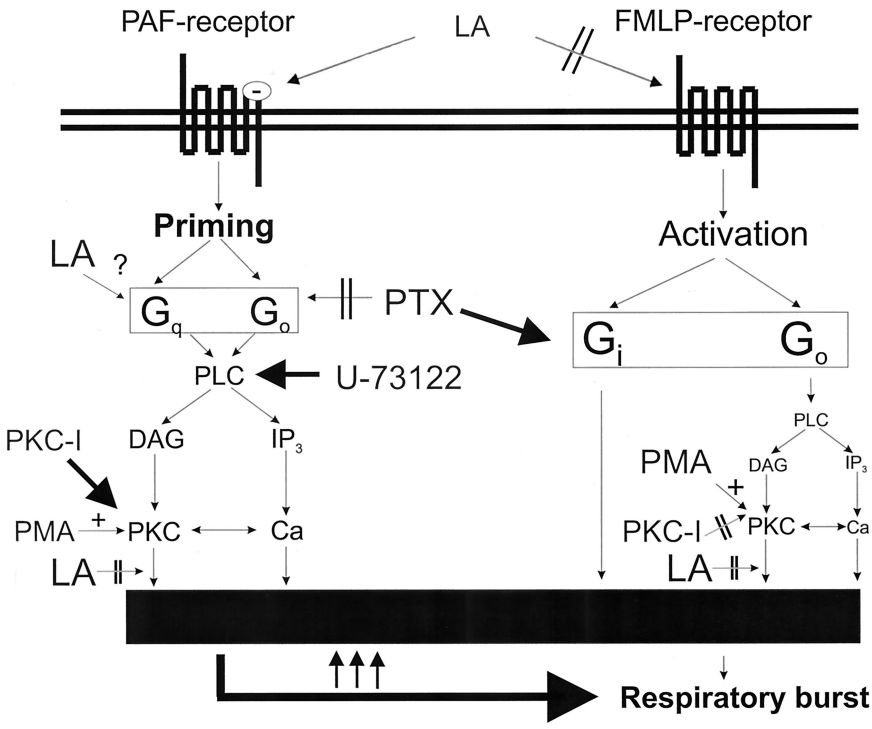

Fig. 6. Schematic overview of our hypothesis of signaling pathways involved in platelet-activating factor (PAF) priming and N-formylmethionine-leucyl-phenylalanine (fMLP) activation of human polymorphonuclear neutrophil respiratory burst (superoxide anion production). Bold arrows indicate inhibition by the specified compound; crossed arrows indicate no effect of the compound. LA = local anesthetic; PTX = pertussis toxin; PLC = phospholipase C; PKC = protein kinase C; DAG = diacylglycerol; IP_3 = inositol trisphosphate; PMA = phorbol myristate acetate. See text for details.

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,[24] acute respiratory distress syndrome,[25] and bacterial or fungal infections.[26] hPMN priming has been shown to be critical for the induction of endothelial injury[27] and lung damage[28] *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*.[1,2] 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 lysophosphatidate-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,[29] 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.[23] 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 G_q proteins selectively.[30] Because we determined that PAF priming is mediated through a PTX-insensitive G protein, we hypothesize that G_q 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 G_i (Durieux ME, unpublished observation, 2000) nor G_o[30] signaling is inhibited by relevant LA concentrations. Although G_s-mediated signaling is PTX-insensitive, it is unlikely to be involved because increases in cyclic adenosine monophosphate levels, resulting from G_s 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 O_2^- 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 O_2^- 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 O_2^- 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.

Pertussis 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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References

1. Hollmann MW, Durieux ME: Local anesthetics and the inflammatory response: A new therapeutic indication? ANESTHESIOLOGY 2000; 93:858–75
2. Sasagawa S: Inhibitory effects of local anesthetics on migration, extracellular release of lysosomal enzyme, and superoxide anion production in human polymorphonuclear leukocytes. Immunopharmacol Immunotoxicol 1991; 13: 607–22
3. Fischer LG, Conrad B, Krumm B, Hollmann MW, Durieux ME: Time-dependent attenuation by lidocaine of respiratory burst in human neutrophils primed with lysophosphatic acid (abstract). Anesth Analg 2000; 90:S405
4. Fischer LG, Krumm B, Conrad B, Hollmann MW, Durieux ME: Lysophosphatidic acid has a priming but no activating effect in human neutrophils (abstract). Anesth Analg 2000; 90:S406
5. Condliffe AM, Kitchen E, Chilvers ER: Neutrophil priming: Pathophysiological consequences and underlying mechanisms. Clin Sci 1998; 94:461–71
6. Nakos G, Pneumatikos J, Tsangaris I, Tellis C, Lekka M: Proteins and phospholipids in BAL from patients with hydrostatic pulmonary edema. Am J Respir Crit Care Med 1997; 155:945–51
7. Rabinovici R, Bugelski PJ, Esser KM, Hillegass LM, Vernick J, Feuerstein G: ARDS-like lung injury produced by endotoxin in platelet-activating factor-primed rats. J Appl Physiol 1993; 74:1791–802
8. Rabinovici R, Yeh CG, Hillegass LM, Griswold DE, DiMartino MJ, Vernick J, Fong KL, Feuerstein G: Role of complement in endotoxin/platelet-activating factor-induced lung injury. J Immunol 1992; 149:1744–50
9. Fink A, Geva D, Zung A, Konichezky S, Eliraz A, Bentwich Z: Adult respiratory distress syndrome: Roles of leukotriene C4 and platelet activating factor. Crit Care Med 1990; 18:905–10
10. Goldsmith JA, Kavanagh BP, Pearl RG: Plasma potentiates the priming effects of endotoxin on platelet activating factor-induced pulmonary hypertension in the rabbit lung. Anesth Analg 1996; 83:242–6
11. Matsumoto K, Taki F, Kondoh Y, Taniguchi H, Takagi K: Platelet-activating factor in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. Clin Exp Pharmacol Physiol 1992; 19:509–15
12. Christman BW, Lefferts PL, Blair IA, Snapper JR: Effect of platelet-activating factor receptor antagonism on endotoxin-induced lung dysfunction in awake sheep. Am Rev Respir Dis 1990; 142:1272–8
13. Zehr KJ, Poston RS, Lee PC, Uthoff K, Kumar P, Cho PW, Gillinov AM, Redmond JM, Winkelstein JA, Herskowitz A: Platelet activating factor inhibition reduces lung injury after cardiopulmonary bypass. Ann Thorac Surg 1995; 59: 328–35
14. Pongracz J, Lord JM: Superoxide production in human neutrophils: Evidence for signal redundancy and the involvement of more than one PKC isoenzyme class. Biochem Biophys Res Commun 1998; 247:624–9
15. Christiansen NO: Pertussis toxin inhibits the FMLP-induced membrane association of protein kinase C in human neutrophils. J Leukoc Biol 1990; 47:60–3
16. Koenderman L, Yazdanbakhsh M, Roos D, Verhoeven AJ: Dual mechanisms in priming of the chemoattractant-induced respiratory burst in human granulocytes: A Ca2+-dependent and a Ca2+-independent route. J Immunol 1989; 142:623–8
17. Simchowitz L, Spilberg I: Generation of superoxide radicals by human peripheral neutrophils activated by chemotactic factor: Evidence for the role of calcium. J Lab Clin Med 1979; 93:583–93
18. Collinsworth KA, Kalman SM, Harrison DC: The clinical pharmacology of lidocaine as an antiarrhythymic drug. Circulation 1974; 50:1217–30
19. Wiklund L: Human hepatic blood flow and its relation to systemic circulation during intravenous infusion of lidocaine. Acta Anaesthesiol Scand 1977; 21:148–60
20. Tsai PS, Buerkle H, Huang LT, Lee TC, Yang LC, Lee JH: Lidocaine concentrations in plasma and cerebrospinal fluid after systemic bolus administration in humans. Anesth Analg 1998; 87:601–4
21. Mayumi T, Dohi S, Takahashi T: Plasma concentrations of lidocaine associated with cervical, thoracic, and lumbar epidural anesthesia. Anesth Analg 1983; 62:578–80
22. Arlander E, Ost A, Stahlberg D, Lofberg R: Ropivacaine gel in active distal ulcerative colitis and proctitis: A pharmacokinetic and exploratory clinical study. Aliment Pharmacol Ther 1996; 10:73–81
23. Sullivan LM, Hoenemann CW, Arledge JAM, Durieux ME: Synergistic inhibition of lysophosphatidic acid signaling by charged and uncharged local anesthetics. Anesth Analg 1999; 88:1117–24

24. Krause PJ, Maderazo EG, Bannon P, Kosciol K, Malech HM: Neutrophil heterogeneity in patients with blunt trauma. J Lab Clin Med 1988; 112:208–15

25. Chollet-Martin S, Montravers P, Gibert C, Elbim C, Desmonts JM, Fagon JY, Gougerot-Pocidalo MA: Subpopulation of hyperresponsive polymorphonuclear neutrophils in patients with adult respiratory distress syndrome: Role of cytokine production. Am Rev Respir Dis 1992; 146:990–6

26. Bass DA, Olbrantz P, Szejda P, Seeds MC, McCall CE: Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. J Immunol 1986; 136:860–6

27. Smedly LA, Tonnesen MG, Sandhaus RA, Haslett C, Guthrie LA, Johnston RBJ, Henson PM, Worthen GS: Neutrophil-mediated injury to endothelial cells: Enhancement by endotoxin and essential role of neutrophil elastase. J Clin Invest 1986; 77:1233–43

28. Worthen GS, Haslett C, Rees AJ, Gumbay RS, Henson JE, Henson PM: Neutrophil-mediated pulmonary vascular injury: Synergistic effect of trace amounts of lipopolysaccharide and neutrophil stimuli on vascular permeability and neutrophil sequestration in the lung. Am Rev Respir Dis 1987; 136:19–28

29. Krause KH, Demaurex N, Jaconi M, Lew DP: Ion channels and receptor-mediated Ca2+ influx in neutrophil granulocytes. Blood Cells 1993; 19:165–73

30. Hollmann MW, Wieczorek KS, Berger A, Durieux ME: Local anesthetic inhibition of G protein-coupled receptor signaling by interference with Galpha(q) protein function. Mol Pharmacol 2001; 59:294–301

31. Richter J: Effect of adenosine analogues and cAMP-raising agents on TNF-, GM-CSF-, and chemotactic peptide-induced degranulation in single adherent neutrophils. J Leukoc Biol 1992; 51:270–5

32. Agwu DE, McCall CE, McPhail LC: Regulation of phospholipase D-induced hydrolysis of choline-containing phosphoglycerides by cyclic AMP in human neutrophils. J Immunol 1991; 146:3895–903

33. Tyagi SR, Olson SC, Burnham DN, Lambeth JD: Cyclic AMP-elevating agents block chemoattractant activation of diradylglycerol generation by inhibiting phospholipase D activation. J Biol Chem 1991; 266:3498–504

34. Rabet L, Coffer PJ, Wolthuis RM, Zwartkruis F, Koenderman L, Bos JL: Differential fMet-Leu-Phe- and platelet-activating factor-induced signaling toward Ral activation in primary human neutrophils. J Biol Chem 1999; 274:21847–52

35. Goldsmith P, Gierschik P, Milligan G, Unson CG, Vinitsky R, Malech HL, Spiegel AM: Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. J Biol Chem 1987; 262:14683–8

36. Uhing RJ, Polakis PG, Snyderman R: Isolation of GTP-binding proteins from myeloid HL-60 cells: Identification of two pertussis toxin substrates. J Biol Chem 1987; 262:15575–9