Effects of Antidepressants on Function and Viability of Human Neutrophils

Danja Strümper, M.D.,† Marcel E. Durieux, M.D., Ph.D.,‡ Markus W. Holland, M.D., Ph.D.,§ Barbara Tröster,# Christel G. den Bakker,** Marco A. E. Marcus, M.D., Ph.D.††

Background: Antidepressants are frequently used in chronic pain therapy and are under investigation as long-acting local anesthetics. Because of the structural similarities between antidepressants and local anesthetics, the authors hypothesized that these compounds act similarly, and they investigated the effects of nortriptyline, amitriptyline, imipramine, and fluoxetine on priming and activation of human polymorphonuclear neutrophils (hPMNs).

Methods: Effects of 30-, 120-, and 240-min preincubation with nortriptyline (10⁻⁷–10⁻⁴ M), amitriptyline (10⁻⁶–10⁻³ M), imipramine (10⁻⁶–10⁻³ M), or fluoxetine (10⁻⁷–10⁻⁴ M) on O₂⁻ generation of platelet activating factor–primed (10⁻⁶ M) and/or formyl-methionyl-leucyl-phenylalanine-activated (10⁻⁶ M) isolated hPMNs were determined. All data are reported as mean ± SD (statistics: t test, *P < 0.05).

Results: Brief incubation in low concentrations of nortriptyline, amitriptyline, or fluoxetine (all at 10⁻⁵ M) did inhibit priming but not activation of hPMNs. Imipramine (10⁻⁵ M) affected neither priming nor activation. Prolonged incubation in lower concentrations of all antidepressants influenced neither priming nor activation. However, at higher concentrations, all four compounds exerted cytotoxic effects: virtually all hPMNs were killed by amitriptyline and imipramine (both at 10⁻³ M) or nortriptyline and fluoxetine (both at 10⁻⁴ M).

Conclusion: Antidepressants, in low concentrations, inhibited priming but not activation of hPMNs. However, at concentrations similar to those attained after local injection, and in marked contrast to local anesthetics, antidepressants are profoundly toxic to hPMNs.

TRICYCLIC antidepressants (TCAs) such as amitriptyline and imipramine are frequently used in the treatment of chronic pain syndromes. The mechanism of action of these compounds when used for this purpose is poorly understood, limiting further development of these compounds for use in chronic pain therapy.

TCAs share many structural similarities with local anesthetics (LAs). Both groups of compounds consist of a hydrophobic portion (usually a single ring structure in LAs and a tricyclic structure in TCAs), linked to an amide via a linear intermediate moiety (an amide or ester linkage in LAs and a hydrocarbon chain in TCAs). Thus, one might anticipate TCAs to share some of the properties of LAs. Indeed, amitriptyline is known to have sodium channel blocking properties and has been studied for use as a long-acting LA. It is therefore conceivable that the beneficial effects of TCAs in the treatment of chronic pain might result from some properties they share with LAs.

One property of LAs that has recently received attention is their antiinflammatory effect. LAs have been shown to inhibit “primed” overactivity of human polymorphonuclear granulocytes (hPMNs) without interfering with their normal function. The major platelet activating factor (PAF)-induced priming pathway is dependent on phospholipase C and protein kinase C and is mainly Galph(q)-mediated, whereas formyl-methionyl-leucyl-phenylalanine (fMLP)-induced activation is Gαi5-mediated. LAs therefore are unique compounds, able to modulate the pathologic inflammatory response without suppressing normal function. This effect has been shown to be profoundly time-dependent, i.e., 5- to 10-fold greater LA potency is attained after incubation for hours to days. Because LAs are clinically often administered for long periods (via the epidural route), this finding may well be of clinical relevance. Furthermore, it has been shown that both LAs and TCAs are able to inhibit fMLP-induced chemotaxis of hPMNs.

Because of their structural similarities to LAs, we hypothesized that TCAs might act in a similar manner, inhibiting an overactivated inflammatory system but without interfering with normal function. Because many chronic pain syndromes have a well-defined inflammatory component, such an effect could potentially explain part of their clinical efficacy in these settings.

Therefore, we studied (as LAs have been tested) the effects of the TCA amitriptyline, its metabolite nortriptyline, the structurally closely related imipramine, and (for comparative purposes) the nontricyclic antidepressant fluoxetine on priming and activation of hPMNs and determined if any effects observed were time-dependent. Our findings demonstrate that profound differences exist between LAs and antidepressants in this regard. Whereas nortriptyline, amitriptyline, and fluoxetine at concentrations like those attained after oral administration did inhibit priming (but not activation) of hPMNs, imipramine had no effect. The effect of the former drugs was not time-dependent. Furthermore, we observed that at concentrations like those attained after oral administration did inhibit priming (but not activation) of hPMNs, imipramine had no effect. The effect of the former drugs was not time-dependent. Furthermore, we observed that at concentrations like those attained after oral administration did inhibit priming (but not activation) of hPMNs, imipramine had no effect. The effect of the former drugs was not time-dependent.
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Materials and Methods

The study protocol was approved by the institutional review board and written informed consent was obtained from each volunteer.

Preparation of bPMNs

Venous blood was obtained from healthy volunteers who had not taken any medication for the previous 2 weeks. Blood was heparinized (10 U/ml) and hPMNs were isolated by a one-step Histopaque separation procedure. After 35 min of centrifugation at 1400 rpm, hPMNs were washed three times with Hank’s balanced salt solution (HBSS; containing 10 U/ml heparin) and were centrifuged after each washing step at 1000 rpm (20 min after the first wash and 10 min after the second and third washes). hPMNs were then resuspended in 5 ml pure HBSS and counted with a hemocytometer.

The Unopette In Vitro Diagnostic System (Becton Dickinson, Franklin Lakes, NJ) was used for enumeration of hPMNs in the suspension, which was then diluted with HBSS to obtain a final hPMN suspension of 5 × 10⁶ cells/ml. The purity of our hPMN suspension, assessed morphologically, exceeded 98%. Its viability, determined by trypan blue exclusion, exceeded 98%. All preparations and assays were performed at room temperature.

Superoxide Anion Generation in bPMNs

We used the cytochrome c reduction assay to measure extracellular superoxide anion (O₂⁻) production by activated hPMNs, as described previously. O₂⁻ generation was measured spectrophotometrically as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c.

The O₂⁻ production was measured by 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 hPMN suspension (final concentration, 10⁶ 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 O₂⁻ can reduce cytochrome c, but only O₂⁻ is destroyed by SOD, by catalyzing its conversion to hydrogen peroxide. By subtracting the absorbance of the SOD reaction (representing cytochrome c reduction caused by other radical oxygen metabolites), the selective contribution of O₂⁻ can be determined. Catalase was used to degrade hydrogen peroxide into hydrogen and oxygen, preventing hydrogen peroxide from reoxidizing the reduced cytochrome c. Such hydrogen peroxide-dependent oxidation will give inaccurate, falsely low results.

The hPMNs were incubated in 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (final concentration, 10⁻⁶ M) for 5 min before activation of the reaction by addition of fMLP (final concentration, 10⁻⁶ M). The change in absorbance at 550 nm was monitored after 16 min (after which O₂⁻ production essentially ceases, as determined in previous experiments⁹). The reference sample was measured immediately after, and O₂⁻-dependent cytochrome c reduction was determined by subtracting the reference value from the study sample value. To test effects of nortriptyline, amitriptyline, imipramine, and fluoxetine (each diluted in HBSS and adjusted to a pH of 7.2) on either priming or activation, hPMNs were incubated for 30, 120, and 240 min, respectively, in various concentrations of the drugs before priming and/or activation of the hPMNs.

Reagents

HBSS (without phenol red, with calcium and magnesium) was obtained from Whittaker Bioproducts (Walkersville, MD), and SOD (from bovine liver), fMLP, cytochrome c (from horse heart), catalase (from bovine liver), PAF, Histopaque-1119, nortriptyline, amitriptyline, imipramine, fluoxetine, and lidocaine were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (protease-free bovine albumin fraction, fatty acid-free) was obtained from ICN Biomedicals, Inc. (Aurora, OH). Polymorph neutrophil isolation medium was obtained from Accurate Chemical & Scientific Corporation (Westbury, NY).

Statistical Analysis

Data are reported as mean ± SD. Leukocyte metabolic activity is reported either as O₂⁻ generation or as percentage change from control. Blood from at least five donors was used for each data point. Statistical analysis was performed with the Student t test or paired t test, as appropriate. P < 0.05 was considered significant. Correlations were determined with use of the Pearson correlation coefficient and regression coefficient. Concentration-response curves were fit to the following logistic function, derived from the Hill equation: y = yₘᵢₓ + (yₘᵢₓ − yₘᵋ)∕[1 + xⁿ∕(xₕ₀ⁿ + xⁿ)], where yₘᵢₓ and yₘᵋ are the maximum and minimum obtained, n is the Hill coefficient, and xₕ₀ is the half-maximal inhibitory concentration (ICₕ₀).

Results

Activation and Priming of bPMNs

To ensure that the properties of our model had not changed as compared with our previous studies,⁹,16 we
determined the ability of PAF to prime fMLP-induced activation of O$_2^-$/H$_2$O$_2$ production in hPMNs. fMLP (10$^{-6}$ M) induced O$_2^-$/H$_2$O$_2$ generation of 2.5 nM/10$^6$ cells. This response was increased 3.6-fold by treatment 5 min prior to activation with PAF (10$^{-6}$ M). Therefore, both activation and priming of hPMNs function appropriately in our model (fig. 1).

Effects of Short-Term Treatment with Antidepressants on Priming and Activation of hPMNs

We next studied the effects of 30-min treatment with 10$^{-5}$ M nortriptyline, amitriptyline, imipramine, and fluoxetine on activation and priming of hPMNs. We have shown in our previous experiments that LAs inhibit neutrophil priming in a concentration-dependent manner at concentrations of 10$^{-6}$ to 10$^{-4}$ M and therefore chose this concentration range for study. The effects of nortriptyline, amitriptyline, imipramine, and fluoxetine on priming and activation are shown in figure 2. Neither compound affected hPMN activation. Nortriptyline and fluoxetine (both 10$^{-5}$ M) inhibited priming to 44$\pm$22% and 45$\pm$15% of control response, respectively. In contrast, imipramine and amitriptyline (both 10$^{-5}$ M) did not affect priming significantly (125$\pm$31% and 84$\pm$8%, respectively).

Figure 3 shows the concentration-response curve of amitriptyline, as a representative antidepressant, for inhibition of priming; calculated IC$_{50}$ was 4.0 $\times$ 10$^{-3}$ M. Maximal inhibition to 11$\pm$8% and 14$\pm$13% of control response was obtained with 0.1 and 1 mM amitriptyline, respectively.

Table 1. IC$_{50}$ Values of Antidepressants for Priming Inhibition in hPMNs

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nortriptyline</td>
<td>7.8</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>40.0</td>
</tr>
<tr>
<td>Imipramine</td>
<td>34.8</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>8.6</td>
</tr>
</tbody>
</table>

hPMNs = human polymorphonuclear neutrophils; IC$_{50}$ = half-maximal inhibition concentration in µM for priming inhibition in hPMNs after 30-min incubation in antidepressants; IC$_{50}$ calculated using Hill equation.
lower hydrophobicity, is associated with a greater inhibitory potency. Calculation with the Pearson equation revealed that the correlation coefficient between logP and percentage inhibition was 0.95. Because fluoxetine is structurally different it was not included in this analysis.

**Long-Term Effects of Antidepressants on Priming and Activation of hPMNs**

We showed in previous experiments that priming inhibition of hPMNs by LAs is time-dependent. For example, 10^-7 M lidocaine has minimal effects on hPMN function after 10-min incubation but inhibits priming by 50% after 4-h incubation. Therefore, we studied the effects of long-term incubation (120 and 240 min) in low concentrations of nortriptyline, amitriptyline, imipramine, or fluoxetine on priming and activation of hPMNs.

The results of the experiments are shown in figure 5. The concentrations chosen were below the calculated IC50: 10^-5 M amitriptyline and imipramine and 10^-6 M nortriptyline and fluoxetine.

Whereas nortriptyline inhibited priming significantly, amitriptyline, imipramine, and fluoxetine did not. Priming inhibition after long-term incubation was not increased with any compound tested, as compared with results obtained after 30-min incubation.

At these concentrations none of the compounds affected hPMNs viability, after either 2- or 4-h incubation (fig. 6). Therefore, amitriptyline, imipramine, and fluoxetine lack any selective effect on activation and/or priming, whereas nortriptyline at lower concentrations inhibits priming but not activation of hPMNs after long-term incubation.
or activation pathway or a general effect on cell viability, we studied their effects on dye exclusion. hPMNs were incubated for 30 min in various concentrations of nortriptyline, amitriptyline, imipramine, or fluoxetine, and cell integrity was determined with the trypan blue vitality test. For example, Figure 7A shows hPMNs incubated for 30 min in HBSS (control). All cells exclude the dye, i.e., they are alive and functional. Figure 7B shows hPMNs incubated in amitriptyline $10^{-3} \text{M}$ for 30 min. No cell is able to exclude the dye, i.e., they are all dead.

Table 3 gives the estimated half-maximal lethal concentrations of the tested compounds. All four compounds were toxic for hPMNs. The half-maximal lethal concentrations are close to those observed for inhibition of activation and priming and make it likely that cellular toxicity explains the observed effects on activation-induced O$_2$ production. Nortriptyline, amitriptyline, imipramine, and fluoxetine killed 100% of hPMNs at concentrations of $10^{-3} \text{M}$ and $10^{-4} \text{M}$, respectively. For comparative purposes, we determined the effect of lidocaine on hPMN viability. Even $10^{-2} \text{M}$ concentrations of lidocaine did not affect PMN viability, as assessed by trypan blue exclusion (data not shown). This lack of hPMN toxicity is similar to our findings in previous studies.9

**Discussion**

Our findings demonstrate that despite structural similarity and similar actions in some models, the effects of LAs and antidepressants on the function of hPMNs are profoundly different. LAs, when applied for brief periods, do not effect activation but block priming with a very shallow concentration-response relationship.9 In contrast, antidepressants have the ability to inhibit both activation and priming, and with a very steep concentration-response curve. The mechanism underlying the inhibition of activation at high concentrations is likely direct toxicity. The mechanisms of this toxicity are not well understood.

Remarkably, both TCAs and selective serotonin reuptake inhibitors were toxic, and at similar concentrations, despite the structural differences between the two groups. LAs, at low concentrations applied for long periods, have no effect on activation but block priming in hPMNs effectively. In contrast, antidepressants (except nortriptyline) at these concentrations exert no effect on either activation or priming of hPMNs. Sacerdote et al.15 observed inhibition of fMLP-induced chemotaxis by TCAs, even at lower concentrations ($10^{-7} \text{M}$). Because we did not determine involved intracellular pathways, we cannot explain this difference; however, a logical hypothesis would be that fMLP-induced chemotaxis and superoxide generation are mediated through different G proteins and signaling pathways (despite coupling to the same receptor), and antidepressants selectively affect the chemotaxis pathway.
ANTIDEPRESSANTS ARE TOXIC TO HUMAN NEUTROPHILS

Table 3. hPMN Viability after Incubation in Antidepressants

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration*</th>
<th>Concentration*</th>
<th>Concentration*</th>
<th>Concentration*</th>
<th>LC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^{-5} M</td>
<td>5 × 10^{-5} M</td>
<td>10^{-4} M</td>
<td>10^{-3} M</td>
<td></td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>7 ± 4</td>
<td>26 ± 8</td>
<td>92 ± 7</td>
<td>ND</td>
<td>61</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>1 ± 1</td>
<td>4 ± 1</td>
<td>6 ± 3</td>
<td>100 ± 0</td>
<td>150</td>
</tr>
<tr>
<td>Imipramine</td>
<td>3 ± 2</td>
<td>29 ± 9</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>52</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>2 ± 1</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>ND</td>
<td>14</td>
</tr>
</tbody>
</table>

hPMN viability determined by trypan blue exclusion after 30-min incubation of hPMNs in antidepressants at various concentrations; control hPMNs were incubated in Hank’s balanced salt solution for 30 min, resulting in 2 ± 2% of cells not excluding dye.

* Mean percentage of dead cells ± SD.

LC50 = half-maximal lethal concentration, calculated using Hill equation; hPMNs = human polymorphonuclear neutrophils; ND = not determined.

Our hPMNs model is an in vitro assay, and the limitations of such a model should be kept in mind. Mechanical manipulation of the hPMNs during purification steps and the lack of interaction with other cell types might affect O2 production. In addition, the protein concentration in the medium is less than in vivo, which could increase the unbound fraction of the compounds tested; therefore, results cannot be extrapolated directly to the clinical setting.

Toxic actions of antidepressants in concentrations as used in this study have been observed previously in cultured rat C6 glioma and human astrocytoma cell lines. Furthermore, the Institute of Occupational Safety and Health published in its Registry of Toxic Effects of Chemical Substances IC50 values for toxicity of imipramine and amitriptyline of 5.4 × 10^{-5} M and 5.6 × 10^{-5} M, respectively (http://iccvam.niehs.nih.gov [December 19, 2002]). However, these effects have not been considered clinically relevant, as such concentrations are not attained during routine clinical use. Therapeutic blood levels of imipramine and fluoxetine for antidepressant use are 0.5–1 × 10^{-6} M. For use in chronic pain states, therapeutic levels are even lower than required for antidepressant effects.

However, this assessment of the relevance of cytotoxic action changes when these compounds are to be used as LAs and injected locally. Under these conditions, concentrations of up to 10^{-2} M are used, 100-fold greater than the concentration that induced 100% cell death in the present study. Thus, cellular toxicity is likely to become relevant in that setting. It is surprising that chronic pain states, therapeutic levels are even lower than required for antidepressant effects.

On the basis of comparable physicochemical properties and protein binding of LAs and TCAs, we estimate similar tissue concentrations after injection. Within the first 30 min, one would expect these to range between the injected concentration and a concentration 10-fold less. Thus, concentrations up to 10^{-3} M can be considered relevant in this setting.

Indeed, the implications of our findings should be considered carefully. First, because the main application of a long-acting LA-like compound would be its use for postoperative analgesia, it should be determined whether injecting a neutrophil toxin around a surgical wound has any influence on wound-healing and infection rates. Second, the concentrations used in local injections are toxic not only for hPMNs but also for neuronal cells. It is conceivable, therefore, that the long-lasting analgesia obtained after injection of amitriptyline might be due to a toxic effect on axonal structures rather than by sodium channel blockade, which has thus far been assumed.

The findings obtained with antidepressants are different from those observed with LAs. Although the antidepressants blocked priming at concentrations somewhat less than those obtained with LAs (lidocaine and bupivacaine inhibited O2 production to 53 ± 5% and 81 ± 4% of initial response at 10^{-4} M, respectively, in this model), we did not observe any effect of LAs on activation. In addition, in contrast to the relatively steep concentration-response relationship for antidepressants, the concentration-response curve for LAs on priming is remarkably flat.

These differences, and the fact that fluoxetine (structurally unrelated to LAs) showed effects similar to those of the TCAs, suggested that the underlying mechanisms of inhibition might be different from those observed with LAs. Our results also indicate that an antiinflammatory action, similar to that described for LAs, is unlikely to be the mechanism underlying the effect of antidepressants in the treatment of chronic pain.

This leaves a number of other avenues to be explored. Effects of antidepressants on potassium channels, α2-δ adrenoceptors,20,21 the endogenous opioid system,22 as well as adenosine A1,23 N-methyl-D-aspartate24 and (amino)butyric acid receptor type B receptors,25 have been reported. Antiinflammatory properties such as activation of the pituitary-adrenocortical axis26, inhibition of nitric oxide release and prostaglandin E2 and hyaluronic acid production in synovial cells27; and inhibition of interleukin-6, interleukin-1β, and tumor necrosis factor-α from human monocytes and of interleukin-2 and interferon-γ from T cells28,29 have also been described.

Our present findings do shed some light on the structure-function relation for blocking of priming by LAs.
The inability of amitriptyline, imipramine, and fluoxetine to inhibit priming, despite long incubation times and their structural similarity with LAs, suggests some molecular properties of LAs that are of importance in priming inhibition. The octanol/water partition coefficients (logP) of nortriptyline (1.17), amitriptyline (2.18), lidocaine (2.26), bupivacaine (3.49), and imipramine (4.80) (http://www.MicroSolvTech.com [December 10, 2002] and http://www.acdlabs.com/publish/physical4.html [July 4, 2002]) are inversely related to the degree of priming inhibition of these compounds.

This suggests that some hydrophobic characteristics might modulate interactions that are required for efficient blocking of hPMN priming. Fluoxetine (logP, 4.52) (http://www.schrodinger.com/Documents/QikPropDataSheet.pdf[2002]) is an exception, which can be explained by the fact that it belongs to a different chemical class. Octanol/water coefficients are reported variably, depending on the analytical method used. Therefore, logP values derived from different sources should be interpreted carefully.

Conclusions

We have shown that imipramine, a TCA with structural similarity to LAs and with several documented LA properties, behaves very different from LAs in its actions on hPMNs. It does not seem to have any selective effects on activation or priming, as shown for LAs. In contrast, it is toxic for hPMNs when applied at concentrations that would be attained routinely if the compound were used for local anesthesia. The TCAs nortriptyline and amitriptyline were also toxic at concentrations used for local injection but inhibited priming in hPMNs at lower concentrations. Similar effects were observed with fluoxetine, an antidepressant with a different structure. These results increase questions about use of these compounds as “LAs” in the clinical setting.

References


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