Annexins I and II bind to lipid A: a possible role in the inhibition of endotoxins

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INTRODUCTION

Endotoxaemia due to Gram-negative bacterial sepsis continues to cause significant morbidity and mortality even after a half-century of antibiotic drug development [1]. Cellular and systemic responses to endotoxin are produced by binding to receptors such as monocyte/macrophage CD14, leading to induction of inflammatory mediators, including tumour necrosis factor-α, interleukin-1β and inducible nitric oxide synthase (iNOS) [2–5]. The active component of endotoxin, lipid A, is a phosphoglycolipid having an acylated and phosphorylated dihexosamine headgroup. Glycosylation of lipid A in the bacterial envelope adds core oligosaccharide and O-specific chain to form lipopolysaccharide (LPS). Deacetylation and dephosphorylation of the lipid A moiety abrogate endotoxic activity, whereas the polysaccharide component contains antigenic determinants but does not contribute to endotoxic activity (reviewed in [4]).

A number of plasma or secreted LPS-binding proteins (LBPs) have been identified that modulate endotoxic potency, suggesting possible strategies for more effectively treating endotoxaemia [1]. The acute-phase reactant LBP binds to the lipid A moiety with high affinity, facilitates the transfer of endotoxin to CD14, and dramatically increases its potency in response induction [3,6,7]. Bactericidal/permeability-increasing protein (BPI), produced by myeloid cells, competes with LBP for LPS/lipid A binding, but, in contrast, acts as an endotoxin antagonist [6,8–10]. Other proteins bind LPS less specifically but still affect endotoxin potency, such as haemoglobin and plasma lipoproteins [11,12].

The annexins are a family of primarily cytosolic, often abundant, calcium-dependent phospholipid-binding proteins. Early studies identified certain annexins as putative glucocorticoid-induced phospholipase A2 (PLA2) inhibitors, ‘lipocortins’. Since then, the topic of annexins as modulators of inflammation and immune function has remained controversial (reviewed in [13–15]). Studies using recombinant partial-length protein, derived peptides and neutralizing antibodies suggest that annexin-I (Anx-I) suppresses inflammatory and immunological responses, including endotoxaemia in rats and LPS-induced iNOS expression in cultured macrophages [2]. Other annexins also appear to have anti-inflammatory properties [16]. These actions of annexins appear to occur in the extracellular compartment. Annexins have been demonstrated on the surfaces of leukocytic and endothelial cells [15,17–19], and soluble annexins are present in blood plasma and bodily secretions [20–28]. Levels of cell-surface and released Anx-I may be regulated by immunomodulatory agents, such as interleukin-1β [29] and glucocorticoids [21,30]. Indeed, the most pronounced effect of glucocorticoids on Anx-I in many cells appears to be increased externalization of the protein rather than increased total cellular content [18,31,32]. However, the process of annexin externalization is not understood. Furthermore, the mechanisms through which annexins might exert anti-inflammatory effects remain unclear. The inhibition of apparent PLA2 activity by annexins in vitro may well reflect substrate sequestration and is of questionable physiological significance [13]. Anx-I has been suggested to suppress monocyte/macrophage and neutrophil function via putative cell-surface receptors [13–15], but these are as yet not well characterized.

Annexin binding to phosphoglycerolipids is relatively nonspecific for headgroup structure, requiring primarily a net negative charge for high-affinity binding [13,33]. Phosphatidylserine (PS), phosphatidylinositol and phosphatic acid are therefore good substrates. Although lipid A is not a glycerolipid, it does have an anionic headgroup. We hypothesize that annexins may bind lipid A in a calcium-dependent manner, possibly modulating its activity or interactions with other endotoxin-binding proteins. The present study demonstrates that annexins exhibit concentration-dependent high-affinity binding to lipid A, compete with LBP for lipid A binding, and inhibit lipid A induction of iNOS assayed by nitrite generation in a cultured system [34,35].

Abbreviations used: Anxl, annexin I; AnxII, annexin II p36 monomer; AnxIII, annexin II p36/p11 tetramer; BPI, bactericidal/permeability-increasing protein; DPLA, diphosphoryl lipid A; EIA, enzyme-linked immunoassay; iNOS, inducible nitric oxide synthase; LBP, lipopolysaccharide; LPS, lipopolysaccharide; LBP, LPS-binding protein; mAb, monoclonal antibody; MPlA, monophosphoryl lipid A; Na/H/CA/BSA, solution containing NaCl, Hepes, CaCl2, and BSA; PLA2, phospholipase A2; PS, phosphatidylserine; RT, room temperature.

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macrophage-like cell line. The data suggest that direct binding of annexins to lipid A may represent a possible mechanism for suppressing cellular and systemic responses to endotoxin.

**MATERIALS AND METHODS**

**Materials**

Annexins were kindly provided by Dr. C. E. Creutz (University of Virginia, Charlottesville, VA, U.S.A.), and purified rabbit LBP was the gift of Dr. P. Tobias (Sciprius Institute, Sandiego, CA, U.S.A.). Diphosphatidyl PS, diphosphoryl lipid A (DPLA) from *Escherichia coli* F583, and LPS from *E. coli* O26:B6 were obtained from Sigma (St. Louis, MO, U.S.A.). Diphosphoryl lipid A from *Salmonella minnesota* R60 (Ra mutant) were from list Biological Laboratories (Campbell, CA, U.S.A.). Monophosphoryl lipid A (MPLA) from *Salmonella minnesota* Re595 was obtained from both Sigma and List. Monomeric molecular masses (Da) are: PS, 736; MPLA, 1682 and DPLA, 1777.

**Preparation of annexins**

Recombinant human Anx-I expressed in *Saccharomyces cerevisiae* and bovine lung annexin II heterotetramer and monomer were purified as previously described [35,36].

**Enzyme-linked immunoassay (EIA) of annexin binding to immobilized lipids**

Coating of microtitre plates was performed essentially as described for assays quantifying LBP and BPI binding to immobilized LPS and lipid A [10,37]. PS, MPLA or DPLA in chloroform was dried under N2, dissolved 1 mg/mL in H2O, vigorously vortexed, and diluted to 40 µg/mL with coating buffer. Lipids (25-50 µl/well) were placed in 96-well microtitre plates (Immulon 2; Dynatech Laboratories, Chantilly, VA, U.S.A.) and incubated overnight at 4°C. Wells were then blocked with 1% (w/v) BSA in 150 mM NaCl/10 mM Hepes (pH 7.0) (Na/H/BSA) for 3 h at room temperature (RT) or overnight at 4°C. Annexins were incubated at the indicated protein and CaCl2 concentrations in 100 µl of Na/H/BSA (Na/H/Ca/BSA) for 1 h at RT. For the experiments represented in Figure 2, 50 x stock solutions of CaCl2 buffered with EGTA were prepared, and final free [Ca2+] used during the annexin incubations. Wells were washed three times with Na/H/Ca, incubated with 100 µl of Na/H/Ca/BSA containing primary monoclonal anti-annexin antibody at 1:5000 dilution for 1.5 h at RT, washed four times, incubated with secondary goat anti-mouse antibody conjugated with peroxidase for 1.5 h at RT, and then washed five times. EIA reactions were performed using the CEA-Roche EIA Reagent Set (Roche Diagnostic Systems, Nutley, NJ, U.S.A.). The final wash was replaced with 75 µl of EIA substrate buffer. Reactions were initiated by adding 75 µl of o-phenylenediamine dihydrochloride (1 tablet/5 ml substrate buffer). Reactions were monitored for 10 min at RT and quenched by adding 75 µl of 4 M HCl, and absorbance was measured at 490 nm.

**TLC of lipid A**

Lipids (60 µg) were spotted on heat-activated silica gel HL plates (Analtech, Newark, DE, U.S.A.) and developed in CHCl3/CH3OH/H2O/30%, aqueous NH4OH (4:4:4:7.5 by vol.). Lipid bands were revealed by spraying with H2O2, scraped, and extracted by sonication in 0.05 M HCl/CH3OH/CHCl3 (1:1:1 by vol.). The aqueous phase was extracted again with CHCl3. The pooled organic phases were washed with 0.05 M HCl, dried under an N2 stream, and sonicated in coating buffer (1.5 ml). EIA was performed as described above.

**Organic extraction and acid hydrolysis of LPS**

LPS (100 µg in 100 µl of H2O) was added to 1.0 ml of 1% (v/v) glacial acetic acid on ice and immediately extracted or submitted to mild acid hydrolysis by heating the mixture at 100°C for 30 min before extraction. Organic-soluble lipid was extracted by adding 1.0 ml of CHCl3/CH3OH (2:1, v/v), vortex mixing and centrifugation for 5 min to separate the phases. The aqueous phase containing intact LPS was discarded. The organic lower phase (0.5 ml) was dried under N2 and sonicated in 200 µl of coating buffer, such that 50 µl of this mixture contained the extract from about 25 µg of the LPS initially added. Serial 1:10 dilutions in coating buffer were made, and 50 µl aliquots were applied to microtitre plates. EIA of Anx-I binding was performed as described above.

**125I-labelled LBP binding assay**

LBP (5-60 µg) in 200 µl of Na/H/3 mM Ca2+ was radioiodinated by incubating with 1 mCi of [125I]NaI (Amersham, Arlington Heights, IL, U.S.A.) and 1-2 Iodobeads (Pierce, Rockford, IL, U.S.A.) for 30 min at RT, and isolated by size-exclusion chromatography on Sephadex G-25 columns. [125I]labelled LBP and Anx-I were mixed in Na/H/BSA/3 mM Ca2+, the protein mixture was applied to lipid-coated and blocked microtitre plates, and it was incubated overnight at 4°C. After three rapid washings with the same buffer, bound protein was solubilized with 1% (w/v) SDS and quantified by gamma counting.

**Nitrite generation in RAW 264.7 cells**

RAW 264.7 cells (American Type Culture Collection, Rockville, MD, U.S.A.) were grown to near-confluency in modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.) in 96-well plates. Annexins were added in 50 µl of fresh serum-free medium, immediately followed by addition of 50 µl of serum-free medium containing LPS or lipid A. The cells were incubated for 24 h at 37°C with the endotoxin/annexin mixture. Nitrite was assayed using Griess reagents [5]. Sulphanilamide (1%, w/v) in 5% (v/v) H3PO4 (50 µl) was added to the medium in the culture wells, which was incubated for 5 min at 37°C; 50 µl of naphthyl-ethylenediamine HCl (0.1%, w/v) was added and incubated for 15 min at 37°C, and absorbance was measured at 570 nm.

**Data presentation and analysis**

Data are presented as the means ± S.E.M. EC50 values reported in the table were derived from the stated number (n) of similar experiments. Representative experiments are shown in all Figures, where the number of replicates per data point are listed.
Error bars smaller than the point symbols were omitted. The significances of differences between groups (P values) were calculated using the unpaired two-tailed t test.

RESULTS

EIA of annexin binding to PS and lipid A

Anx-I in the presence of 1–3 mM Ca^{2+} bound to immobilized PS with an EC_{so} of 3.2 ± 1.5 nM Anx-I (n = 5; Figure 1A). DPLA and MPLA bound Anx-I with similar affinities. The Anx-I EC_{so} for binding to these lipids ranged from 1 to 10 times that seen for PS binding in several parallel experiments (Figure 1A). The amounts of PS and lipid A used to coat the microtitre plates in these experiments were sufficient to saturate Anx-I binding, with maximal binding at ≥ 0.1–1 µg/well (140–1400 pmol) PS, ≥ 0.1–0.3 µg/well (59–180 pmol) MPLA, and ≥ 0.03–0.1 µg/well (17–56 pmol) DPLA. Decreasing the amounts of lipids resulted in lower maximal signals with no change in half-maximal Anx-I concentrations (results not shown).

The calcium requirements for Anx-I binding to PS and lipids A were compared. Anx-I binding to PS was half-maximal at 56 ± 15 µM Ca^{2+} (n = 5; Figure 2A). The calcium concentrations required for binding to commercial lipid A preparations depended upon both the type and the source of the lipid (Figure 2A): the Ca^{2+} EC_{so} for the List products was 9–17 µM Ca^{2+} for DPLA (n = 2) and 258 ± 102 µM for MPLA (n = 3), whereas the Ca^{2+} EC_{so} for the Sigma products was 2.0 ± 0.6 mM Ca^{2+} for...
Anx-I binding to LPS

Anx-I or Anx-II, at concentrations giving maximal binding to PS or lipid A, usually did not show significant binding to either *E. coli* O26:B6 LPS (Figure 1) or to *S. minnesota* Ra mutant LPS, which contains core oligosaccharide but lacks the O-specific component (results not shown). The molar amounts of O26:B6 LPS (130 pmol) and Ra LPS (500 pmol) applied to the wells were similar to the amounts of phospholipids which gave maximal Anx-I binding (see the previous section). In contrast, plates coated with LPS or lipid A bound similar amounts of radioiodinated LBP (results not shown), indicating that the inability to detect annexin binding to LPS-coated plates was not due to an insufficient amount of adherent LPS. Some degree of LPS binding by annexin I or II was occasionally detected at higher concentrations of protein or Ca²⁺, conditions also associated with increased lipid-independent binding (Figure 1B). To address the possibility that annexins may bind to LPS but dissociate during the antibody incubation and washing steps of the EIA, the binding of ¹²⁵I-labelled Anx-I to LPS-coated plates was examined with three rapid washes at 4 °C after incubation. This technique demonstrated PS but not LPS binding by ¹²⁵I-labelled Anx-I at concentrations up to 400 nM (results not shown). It is possible that annexin binding to LPS, when observed, was at least in part due to lipid A or other phospholipid impurities in the LPS preparations. Table 1 demonstrates the presence of organic-extractable Anx-I-binding activity associated with LPS. Mild acid hydrolysis, which generates lipid A from LPS, increased the amount of this activity.

Anx-I inhibits LBP binding to lipid A

Since LBP binding to lipid A is important in producing the physiological effects of endotoxin [3,6,7], the ability of Anx-I to inhibit LBP binding was investigated (Table 2). Nanomolar concentrations of ¹²⁵I-labelled LBP bound to immobilized lipid A but showed relatively little binding to PS, consistent with previous observations that LBP has a low affinity for phosphoglycerolipids [7]. An approx. 10-fold molar excess of Anx-I inhibited ¹²⁵I-labelled LBP binding to DPLA, MPLA and PS by over 50%. Conversely, LBP inhibited radioiodinated Anx-I binding to lipid A but not to PS (results not shown).

Annexins inhibit lipid A-stimulated nitrate generation

Endotoxin-stimulated nitrate production reflects increased iNOS expression in RAW 264.7 macrophage-like cells [5]. These cells were used to investigate whether annexin binding affected responses to lipid A and LPS (Figure 3). DPLA-dependent

Table 1 Anx-I binding to organic extracts of LPS

LPS was extracted with CHCl₃/MeOH either before or after mild acid hydrolysis. Organic-soluble material was coated onto microtitre wells in amounts corresponding to the indicated amount (µg) of the starting LPS. Anx-I (50 nM) binding in the presence of 3 mM Ca²⁺ was determined by EIA as described in the Materials and methods section. n = 3 wells/data point. *P < 0.002 compared with before acid hydrolysis.

<table>
<thead>
<tr>
<th>LPS organic extract (relative amount)</th>
<th>A₁₀₀ before acid hydrolysis</th>
<th>A₁₀₀ after acid hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.001 ± 0.011</td>
<td>0.005 ± 0.008</td>
</tr>
<tr>
<td>2.5</td>
<td>0.099 ± 0.013</td>
<td>0.218 ± 0.006</td>
</tr>
<tr>
<td>25</td>
<td>0.802 ± 0.025</td>
<td>1.015 ± 0.023</td>
</tr>
</tbody>
</table>

Table 2 Inhibition of LBP binding to lipid A by Anx-I

¹²⁵I-labelled LBP (40 nM) in the absence (−) or presence (+) of 550 nM Anx-I was incubated with 3 mM Ca²⁺ in microtitre wells coated with no lipid, PS, MPLA or DPLA. Bound radioactivity was determined as described in the Materials and methods section. n = 4 wells/data point. *P < 0.001 compared with no Anx-I.

<table>
<thead>
<tr>
<th>LBP binding (fmol/well)</th>
<th>Anx-I</th>
<th>No lipid</th>
<th>PS</th>
<th>MPLA</th>
<th>DPLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>1.31 ± 0.04</td>
<td>2.89 ± 0.23</td>
<td>19.67 ± 0.43</td>
<td>11.59 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>1.19 ± 0.02</td>
<td>1.90 ± 0.17</td>
<td>10.36 ± 0.43*</td>
<td>4.52 ± 0.37*</td>
<td></td>
</tr>
</tbody>
</table>

![Figure 3 Effects of annexins on lipid A- and LPS-induced nitrite generation in RAW 264.7 cells](image)
nitrite generation was reduced by 50–70% in the presence of Anx-I, Anx-II p36 monomer (Anx-IIIm) or Anx-III. Similarly, Anx-I (10 μg/ml) caused a 50% inhibition of nitrite generation stimulated by DPLA (1 μg/ml) in C6 glioma cells (results not shown). In contrast, none of the annexins consistently inhibited nitrite production stimulated by LPS.

**DISCUSSION**

The present study suggests that annexins might exert anti-inflammatory effects by binding directly to lipid inflammatory mediators, thereby inhibiting their interactions with cellular receptors or accessory binding proteins. Kinetic studies have shown that, in the presence of calcium, the dissociation of annexins from bound phospholipid is quite slow [40]. This property allowed the development of an enzyme-linked immunoassay (EIA) based on the calcium-dependent binding of annexins to lipids immobilized on microtitre plates. The EIA demonstrated the binding of Anx-I and Anx-III to endotoxin lipid A at low micromolar protein and submillimolar Ca²⁺ binding of Anx-I and Anx-II to endotoxin lipid A at low to lipids immobilized on microtitre plates. The EIA demonstrated the binding of Anx-I and Anx-III to endotoxin lipid A at low micromolar protein and submillimolar Ca²⁺ concentrations. For comparison, binding to PS was also examined, since annexin binding to this lipid has been well characterized using other assays. The affinity of Anx-II for PS liposomes is about 1 nM [41], similar to the EC₅₀ obtained here. The Ca²⁺ EC₅₀ for Anx-I binding to PS was about 56 μM using the present assay, whereas values of 1.3–30 μM have been obtained using vesicle or liposome suspensions (summarized in [13]).

It is likely that annexins bind lipid A in a manner similar to that described for phosphoglycerolipids, involving both electrostatic and hydrophobic interactions between Ca²⁺, anionic phospholipid and binding domains of the protein [33,42]. In this model, addition of the LPS polysaccharide chain might be predicted to hinder sterically close approach of the annexin molecule to the lipid A headgroup, which could explain why annexin binding to LPS was not definitively seen under the conditions used here. LBP and the structurally related (45% homology) BPI are soluble proteins that also bind lipid A through ionic and hydrophobic interactions [8,43], and the competition data (Table 2) suggest that the binding sites of the annexin and LBP molecules overlap. However, unlike annexins, LBP and BPI bind phosphoglycerolipids rather poorly (Table 2) [7,43] and bind purified LPS as well as lipid A with high (nM) affinities [6–8,10]. It thus appears that the binding conformations of LBP and BPI accommodate the polysaccharide chain, whereas those of annexins do not do so. It should be noted that the ability of the proteins to bind LPS may depend on the milieu in which LPS is presented. For example, the affinity of BPI for LPS in intact bacteria appears to be dependent on the polysaccharide chain length (nM for short-chain smooth forms compared with μM for long-chain rough forms) [8,44], whereas binding to purified LPS and lipid A is much less affected by chain length [8,10]. Additional studies will be needed to determine whether annexin binding to LPS can be reproducibly demonstrated under conditions other than those used here.

Annexin binding to lipid A inhibits nitrite generation in RAW 264.7 cells (Figure 3). A previous study found that recombinant human Anx-I-(1–188) fragment markedly inhibited LPS-stimulated NOS induction and nitrite generation in J774.2 macrophage-like cells [2]. However, that study did not determine that annexin bound to LPS, and the authors suggested that this inhibition might be mediated more indirectly via an inhibitory cellular annexin receptor. The present study did not confirm a reproducible effect of Anx-I on responses to LPS (Figure 3). It may be that Anx-I-(1–188) binds LPS whereas full-length Anx-I does not; or, our Anx-I does not bind to a putative inhibitory cell-surface Anx-I receptor [14,15] on RAW 264.7 cells, either because the cells lack the receptor or because of improper protein conformation [15]. In any case, the equivocal effect of annexins on responses to LPS in the present study argues that the observed inhibition of lipid A effects is due to annexin binding to the lipid rather than to a cellular annexin receptor.

An important point raised by our data is that the composition of endotoxin preparations may influence annexin binding and thus may complicate the interpretation of experimental results. LPS preparations may contain an organic-extractable component that binds annexins (Table 1). We have not further characterized this material, but presumably it consists of bacterial membrane phospholipids, possibly including lipid A. Annexin binding to these LPS preparations may modify the biological activity of the lipid A component and could have other effects, such as aggregation of LPS-containing vesicles, which might also influence endotoxic properties even without direct binding to LPS. Furthermore, lipid A preparations may contain impurities that inhibit Ca²⁺-dependent annexin binding (Figure 2).

The physiological significance of annexin binding to endotoxin remains to be determined. The concentrations of Ca²⁺ (≈ 2 mM) and annexins in extracellular fluids are compatible with endotoxin binding in vivo. In healthy humans, plasma levels of annexins have been reported as approx.: Anx-I, 1.2 nM [28] and 0.9 nM [21]; Anx-I + II, 0.5 nM [26]; Anx-V, 0.18 nM [26]. 0–0.14 nM [20] and 0.05 nM [22]. Annexins II, III, IV and VI have also been detected in normal human plasma [26,27]. Annexins are present in bronchoalveolar [24,25,30,45], intestinal [24], biliary [25] and prostatic secretions, at concentrations of 4 μM Anx-I and 1.8 μM Anx-V in the latter [23]. Annexin levels in blood and secretions may be increased after administration of glucocorticoid [30] with a 3-fold rise seen in human serum Anx-I [21], and during various disease states, sometimes by an order of magnitude or more [22,24,26,27]. Annexin levels are markedly increased in tissues adjacent to areas of inflammation and injury [38,46]. Thus annexin synthesis and release may represent a mechanism to contain or limit inflammatory responses. Annexins have the potential to inhibit the effects of endotoxin by interfering with the formation of LBP/lipid A complexes (Table 2) or by blocking cellular responses to lipid A (Figure 3). A critical question is whether endogenous annexins can bind to or modulate responses to LPS under in vivo conditions, or whether these interactions are limited to lipid A. Furthermore, since the potency of endotoxin in vivo is highly dependent on complex interactions with multiple binding proteins [6–12], the efficacies of annexins at physiological concentrations of endotoxin and its binding proteins must be established. Finally, how annexins might interact with heterogeneous composites of LPS, lipid A and membrane phospholipids produced during bacterial infections is unknown. The previous finding that dexamethasone suppression of LPS-stimulated endotoxaemia in rats was reversed by neutralizing antibodies against Anx-I [2] provides some evidence that Anx-I may indeed have a physiological role.

The suppression of inflammatory responses by annexin binding to lipid mediators may not be limited to endotoxin, as annexin V has been shown to bind platelet-activating factor [33]. Whether this leads to antagonism of platelet-activating factor receptor binding and functional responses has yet to be established. Overall, it appears that annexins have the potential to modulate inflammatory and immunological processes at multiple levels [13–15], some of which may be affected by any annexin and others which may be protein specific. The data presented here underscore the possible utility of annexins or derived peptides as therapeutic agents for the treatment of endotoxaemia.
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