Binding and Neutralization of Lipopolysaccharides by Plant Proanthocyanidins

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Proanthocyanidins (PACs), polyphenolic metabolites that are widely distributed in higher plants, have been associated with potential positive health benefits including antibacterial, chemotherapeutic, and antiatherosclerotic activities. In this paper, we analyze the binding of PACs from cranberries, tea, and grapes to lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria and the cause of several human illnesses. We demonstrate that in the case of cranberries, the most potent LPS-binding activity is contained within a PAC fraction composed of polymers with an average degree of polymerization of 21. The PAC fraction modestly inhibits the binding of LPS to the surface of HEK 293 cells expressing the full complement of LPS receptors (TLR4/MD2 and CD14), while it significantly abrogates the endocytosis of LPS. This PAC fraction also inhibits LPS-induced nuclear factor-κB activation in a manner that is not readily overcome by excess LPS. Such an effect is mediated through the inhibition of LPS interaction with TLR4/MD2 and the partial abrogation of LPS interaction with CD14. Importantly, PAC concentrations that mediate effective LPS neutralization elicit minimal *in vitro* cytotoxicity. Our results identify PACs as a new class of LPS-binding compound and suggest that they have potential utility in applications that necessitate either the purification and removal of LPS or the *in vivo* neutralization of LPS.

Proanthocyanidins (PACs) are plant-derived polyphenolic compounds composed of flavanoid subunits and have recently been associated with several potential positive health benefits. For example, PACs have been shown to possess cardioprotective properties through the inhibition of both LDL oxidation and platelet aggregation.[1] PACs have also been shown to have antioxidant properties, scavenging free radicals in biological systems.[2] Of particular note is the observation that PACs from cranberries are effective in the mitigation of urinary tract infections through the decreased adhesion of pathogenic bacteria to uroepithelial cells.[3–6] Detailed studies have attributed this activity to PACs with a degree of polymerization of 4 to 5 containing at least one unique interflavan subunit linkage consisting of one carbon–carbon and one carbon–oxygen bond (referred to as an A-type bond).[7] More recently, it has been shown that PACs induce conformational changes in bacterial P-fimbriae that reduce the adhesive forces between these proteins and epithelial cell surface receptors.[8] Recent work in our laboratory pointed to still further activities of high molecular weight polymers from cranberry juice that inhibited the nonspecific adhesion of bacteria to a protein-functionalized immunosensor surface.[9] On the basis of this observation, we were prompted to investigate the potential for previously undescribed interactions of cranberry juice components with other molecules comprising the bacterial cell surface.

Lipopolysaccharide (LPS), the major component of the outer cell membrane of Gram-negative bacteria, is the primary cause of sepsis, an inflammatory syndrome characterized by an overwhelming systemic response to bacterial infection. Sepsis has become the most common cause of death in intensive care units in the United States, with 120 000 deaths annually and associated health-care costs of $16.7 billion.[10] Commonly referred to as bacterial "endotoxin", LPS is composed primarily of three domains: (1) a bacterial membrane-proximal lipid A moiety, (2) a core oligosaccharide region, which connects to (3) the O-antigen, a branched polysaccharide that extends from the core oligosaccharide.[11] LPS present in blood binds to LPS-binding protein,[12] which transfers LPS to the membrane-anchored receptor, CD14, on mononuclear macrophages. CD14 then mediates the interaction of LPS with the bipartite receptor complex, Toll-like receptor 4/MD2 (TLR4/MD2), resulting in intracellular signaling and production of nuclear factor-κB (NF-κB)-activated inflammatory cytokines.[13]

Given the important role of LPS in the onset of sepsis, much effort has been focused on the isolation of robust LPS-binding compounds. The ultimate application of these compounds ranges from the purification and removal of LPS from solutions where its presence is not desirable (e.g., from pharmaceutical preparations) to the *in vivo* neutralization of LPS in septic patients. This pursuit has resulted in the identification of several classes of compounds possessing desirable LPS-binding characteristics such as the cyclic decapeptide polymyxin B[14] and the polyamine spermine and its structural analogues.[15] Polymyxin B, which has a moderately high affinity for LPS ($\sim$0.4 μM),[14] has been used for the successful removal of LPS from tissue culture media[16] and blood.[17] However, its *in vivo* applications remain limited due to its high toxicity. Polyamines such as spermine are often limited in their specificity, as the mode of recognition is largely electrostatic. Hence, the need for the identification of alternative LPS-binding substances remains.

In the present study, we report the first description of the LPS-binding properties of PACs from cranberries, tea, and grapes. Focusing more closely on PACs from cranberries, we demonstrate the binding of LPS from multiple bacterial species with an apparent affinity for LPS that is comparable to that reported for polymyxin B.[14] The recognition of LPS by PACs appears to be mediated largely through interaction with the conserved lipid A moiety. We also demonstrate the ability of PACs to inhibit the interaction of LPS with cells expressing the full complement of LPS receptors. PACs inhibit LPS interaction with mammalian cells largely through abrogation of LPS interaction with TLR4/MD2, an activity that also mediates the inhibition of LPS-induced NF-κB activation. This is the first report of the LPS-binding activity of PACs, and we discuss our findings in the context of the potential utility of PACs for endotoxin purification and removal or the *in vivo* treatment of sepsis.

Results and Discussion

PACs from Multiple Sources Bind to LPS. PACs are naturally occurring plant-derived polymers composed chiefly of the

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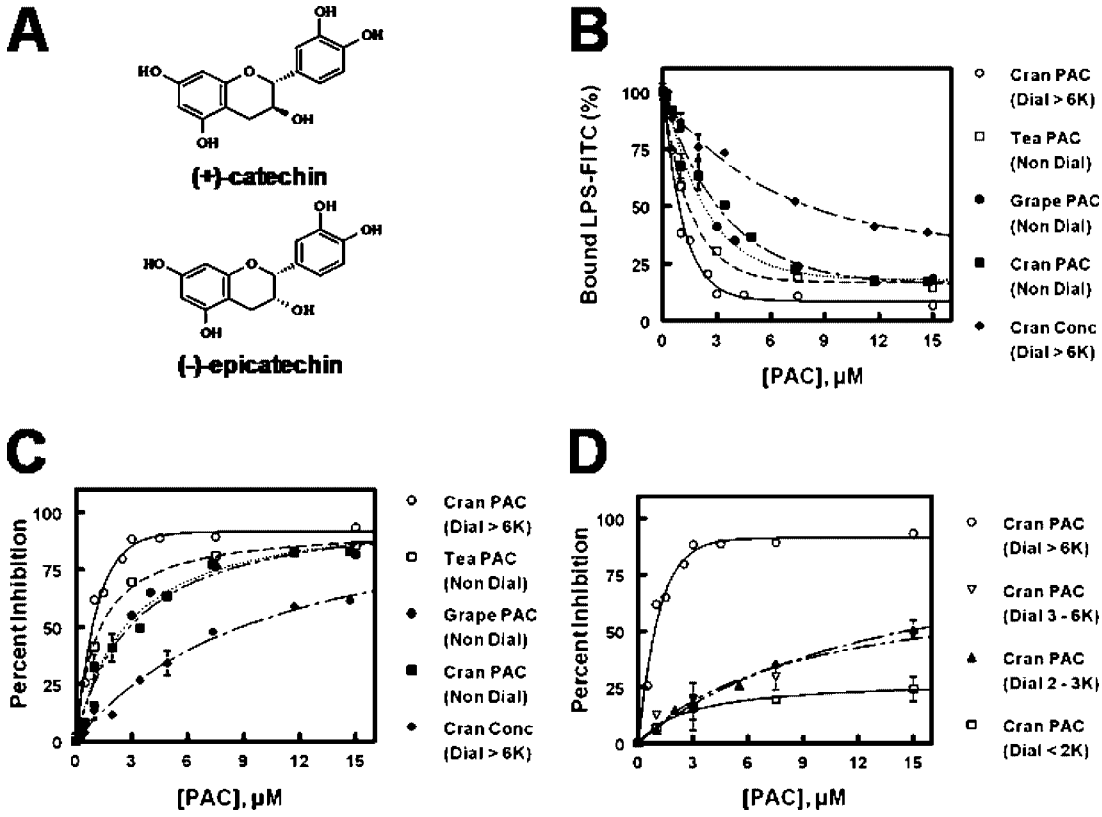

Figure 1. Interaction of PACs with LPS. (A) PACs are polymers commonly composed of (+)-catechin and (–)-epicatechin flavanoid subunits. (B) The data show the percentage of LPS bound to immobilized polymyxin B after co-incubation of LPS with PACs from cranberry, tea, and grapes. The LPS-binding activity of PACs from all three sources is concentration-dependent. (C) The data in (B) are presented as percent inhibition. (D) For cranberry PACs, the majority of the LPS-binding activity is contained within the fraction composed of polymers > 6000 MW (average degree of polymerization of 21). Data in panels B–D are the mean ± standard deviation and are representative of triplicate experiments. PAC concentrations are reported in tannic acid equivalents as described in the Experimental Section.

monomeric flavan-3-ol subunits (+)-catechin and (–)-epicatechin and their derivatives (Figure 1A). Intersubunit linkages are most commonly single intermolecular bonds between carbon atoms (B-type). In some PAC species, however, subunits are linked by two intermolecular bonds; one carbon–carbon and one carbon–oxygen (A-type). Previous work has established the interaction of PACs with protein components of the bacterial cell surface. Foo et al. demonstrated that Sephadex LH20-purified PACs from cranberries inhibited the adherence of P-fimbriated *E. coli* to surfaces containing α-Gal(1→4)*β*-Gal receptor sequences.[7] This activity was associated with PACs with a degree of polymerization of 4 to 5 bearing at least one A-type linkage. Howell et al. later reported that this effect was specific to A-type linkages, as B-type-linked PACs from various sources did not mediate the effect.[18] More recently, Liu and co-workers proposed that PACs decreased bacterial adhesion by altering the P-fimbriae proteins. This theory was based on atomic force microscopy (AFM) studies that showed that PACs induced a shortening of the P-fimbriae, resulting in reduced adhesive forces between the bacterium and the AFM probe tip.[8]

Using a solid-phase binding assay, we assessed the ability of both A- and B-type PACs to bind LPS by determining their ability to inhibit the interaction of *E. coli* LPS with immobilized polymyxin B. Binding experiments comparing PACs from cranberries (which possess both A- and B-type linkages) to those from tea and grapes (which possess exclusively B-type linkages) demonstrated that PACs bearing both linkages bind to LPS in a dose-dependent manner (Figure 1B). Control experiments demonstrated that the decrease in fluorescence signal was not due to the quenching of the LPS-conjugated fluorescein; similar results were obtained using an unlabeled LPS that was subsequently detected with a fluorescein-labeled anti-LPS antibody after soluble PAC:LPS complexes were removed via washing (Figure S-1). Additionally, the interaction of PACs with LPS was specific. When PACs were covalently conjugated to agarose beads, soluble PACs inhibited the binding of LPS to the immobilized PACs on the solid phase (Johnson, unpublished results). When the binding data were plotted as percent inhibition, cranberry PACs that had been enriched through dialysis to contain polymers of larger molecular weight exhibited the most potent LPS-binding activity with an IC_{50} of 0.7 μM (Figure 1C). PACs from tea (nondialyzed) were the next most active, with an IC_{50} of 1.1 μM. Nondialyzed PACs from grapes and cranberries exhibited comparable relative affinities for LPS ($IC_{50} = 3.0$ μM), whereas dialyzed cranberry concentrate (not enriched for PACs) exhibited the lowest relative affinity for LPS ($IC_{50} = 10.5$ μM). A comparison of the LPS-binding activities of cranberry PACs produced by differential dialysis revealed a positive correlation between the relative affinity for LPS and PAC molecular weight. Indeed, the larger molecular weight polymers exhibited higher LPS-binding activity compared to the lower molecular weight PACs (Figure 1D). The cranberry PAC fraction containing, on average, polymers of greater than 6000 MW was used in all subsequent experiments. This fraction was determined to have an average degree of polymerization of 21 and is referred to henceforth as "cranberry PACs". The average degrees of polymerization for the various fractions tested are presented in Table 1.

The LPS-binding activity of cranberry PACs was not limited only to *E. coli* LPS. Comparable affinities were noted for LPS preparations from *Salmonella, Shigella,*and *Pseudomonas*. Further, cranberry PACs were found to bind to two LPS mutants bearing shorter polysaccharide chains of varying lengths (an Ra mutant from *E. coli* and an Rc mutant from *Salmonella*) with only a 3-fold lower affinity relative to wild-type LPS. These results are summarized in Table 2.

Table 1. Average Degree of Polymerization of Size-Fractionated LH20 PACs from Cranberry

LH20 PAC fraction	average DP[a]
non-size-fractionated	12.6
<2K	3.2
2K–3K	5.4
3K–6K	12.9
>6K	21.4

[a] DP = degree of polymerization.

Table 2. Binding of Cranberry LH20 PAC[a] to LPS and Lipid A

bacterial species	apparent IC_{50} (μM)[b]
LPS	
Escherichia coli	0.7 ± 0.2
Salmonella minn.	1.2 ± 0.3
Shigella flexneri	1.6 ± 0.3
Escherichia coli EH 100 (Ra mutant)	2.1 ± 0.7
Salmonella minn. (Rc mutant)	2.1 ± 0.5
Pseudomonas aeruginosa	3.4 ± 1.0
Lipid A[c]	
Escherichia coli	0.3 ± 0.1

[a] Corresponds to PACs of greater than 6000 molecular weight as described in the Experimental Section. [b] Apparent IC_{50}'s are shown with their corresponding 90% confidence intervals. [c] Diphosphoryl form of lipid A.

The apparent affinity of ~0.7 μM observed for cranberry PACs compares favorably with the well-established LPS-binding compound, polymyxin B, which recognizes LPS with an apparent affinity of ~0.4 μM.[14] Polymyxin B has been shown to bind to LPS primarily through electrostatic interactions with the phosphate groups of the lipid A moiety.[19] In our study of the interaction of cranberry PACs with LPS, we also observed a predominant interaction with the lipid A moiety. Cranberry PACs efficiently inhibited the binding of *E. coli* lipid A to polymyxin B with an apparent affinity of 0.3 μM (Table 2). These results confirm the importance of the lipid A moiety in the recognition of LPS.

In contrast to the nature of the interaction of PACs with bacterial P-fimbriae proteins, our findings point to several differences with respect to LPS recognition. First, LPS binding is not specific to the A-type interflavan linkage. PACs from cranberries contain both A- and B-type interflavan subunit bonds,[7,20] while tea and grape PACs contain exclusively B-type interflavan linkages, yet PACs from all three sources efficiently bind LPS. Second, data obtained for cranberry PACs demonstrated that larger polymers (average degree of polymerization 21) possess the highest degree of LPS-binding activity. Further, while other studies on the antibacterial adhesion properties of PACs have concentrated almost exclusively on PACs' effects on uropathogenic *E. coli*, our data clearly show that cranberry PACs bind LPS from multiple Gram-negative bacterial species, largely through recognition of the conserved lipid A moiety.

PACs Slightly Inhibit Membrane Binding of LPS and Significantly Inhibit LPS Endocytosis. Beyond the mere binding of LPS, a desirable attribute of LPS-binding compounds is the ability to inhibit LPS interaction with LPS-responsive mammalian cells. On the basis of their potent LPS-binding activity, we reasoned that cranberry PACs could potentially inhibit LPS interaction with cells expressing the full complement of LPS receptors. Cellular binding studies performed in HEK 293 cells expressing CD14 and Toll-like receptor 4/MD2 (HEK-CD14-TLR4/MD2) revealed a distinct staining pattern corresponding to membrane-bound LPS (Figure 2A, frame "LPS") with minimal nonspecific binding (Figure 2A, frame "No LPS"). While co-incubation of LPS with lipid A did not significantly reduce LPS membrane binding, the presence of cranberry PACs resulted in a modest but significant decrease (~15%) in the amount of membrane-bound LPS (Figure 2C). An anti-TLR4 function-perturbing antibody also caused a modest decrease in LPS binding (~23%), while this same antibody in combination with cranberry PACs did not impart any further LPS-binding perturbation. We found that an anti-CD14 function-perturbing antibody mediated the largest degree of LPS-binding inhibition (~84% inhibition), demonstrating the highly important role of CD14 in LPS membrane binding. Analysis of LPS internalization demonstrated that lipid A and cranberry PACs significantly inhibited endocytosis of LPS (Figure 2B) with degrees of inhibition of 84% and 76%, respectively (Figure 2D). The anti-TLR4 antibody mediated ~50% inhibition of LPS endocytosis, while co-incubation of the antibody with cranberry PACs increased this inhibition further to ~62%. The anti-CD14 antibody mediated approximately 80% inhibition of LPS endocytosis. In control experiments, the addition of AlexaFluor 647-labeled transferrin, a marker of the endocytotic pathway, to the culture medium containing PACs and LPS showed that PACs had no inhibitory effect on normal endocytosis, as a robust staining of the endosomal compartment was observed in both the presence and absence of PACs (Figure S-2). Thus, PACs specifically inhibited the endocytosis of LPS while having no inhibitory effect on overall endocytosis.

In the current model of cellular interaction with LPS, LPS-binding protein (LBP) present in serum binds to and presents LPS to the membrane-resident receptor CD14, which in turn transfers LPS to the bipartite receptor complex, TLR4/MD2.[13] MD2 is the LPS-binding unit of the receptor, while TLR4 serves as the signal transduction component.[13,21,22] The TLR4/MD2-LPS complex ultimately undergoes endocytosis involving a caveolae-dependent uptake mechanism as part of LPS-induced receptor down-regulation.[23] While debate currently exists as to whether TLR4 physically contacts LPS, it is clear that TLR4/MD2 and LPS form a stable complex on the cell surface and that LPS binding to MD2 is a prerequisite for TLR4 signaling activity[24] and LPS endocytosis.[23,25] We hypothesized, therefore, that cranberry PACs inhibit LPS endocytosis by inhibiting LPS interaction with the TLR4/MD2 complex.

PACs Abrogate LPS Interaction with CD14 and TLR4/MD2 but Not LPS-Binding Protein (LBP). Binding studies were performed in order to address the effect of cranberry PACs on LPS interaction with its cognate receptors. Figure 3A shows the results of binding experiments conducted to measure the ability of cranberry PACs to inhibit the binding of *E. coli* LPS to immobilized LBP, CD14, or TLR4/MD2. It was apparent that cranberry PACs had no significant effect on LPS interaction with LBP, while they achieved a maximum inhibition of 38% of LPS binding to CD14 at the highest PAC concentration tested (500 nM). Over the same concentration range, cranberry PACs completely inhibited LPS interaction with TLR4/MD2, with an IC_{50} of ~20 nM PAC. We further found that in the presence of soluble CD14, the amount of LPS bound by TLR4/MD2 was increased approximately 4-fold (Figure 3B), consistent with the established role of CD14 in mediating the transfer of LPS to TLR4/MD2.[26,27] The degree to which cranberry PACs inhibited LPS binding to TLR4/MD2, however, remained unchanged, demonstrating the ability of PACs to inhibit the CD14-mediated transfer of LPS to immobilized TLR4/MD2 (Figure 3B, inset).

PACs Inhibit LPS-Induced NF-κB Activation. On the basis of the LPS-binding activity of cranberry PACs and their abrogation of LPS interaction with cell surface receptors, we reasoned that PACs could also inhibit the LPS-induced activation of the transcription factor, NF-κB. NF-κB activation by LPS leads to the expression of proinflammatory cytokines, resulting in the metabolic and physiologic changes that ultimately lead to pathological conditions, including sepsis.[28] As shown in Figure 4A, cranberry PACs inhibited the activation of NF-κB in a dose-dependent manner in HEK-CD14-TLR4/MD2 cells stimulated with 2 nM LPS with an IC_{50} of 25 nM PAC. Further, the data in Figure 4B show that this inhibition was not readily overcome by an excess of LPS. In the absence of cranberry PACs, an increase in LPS resulted in a

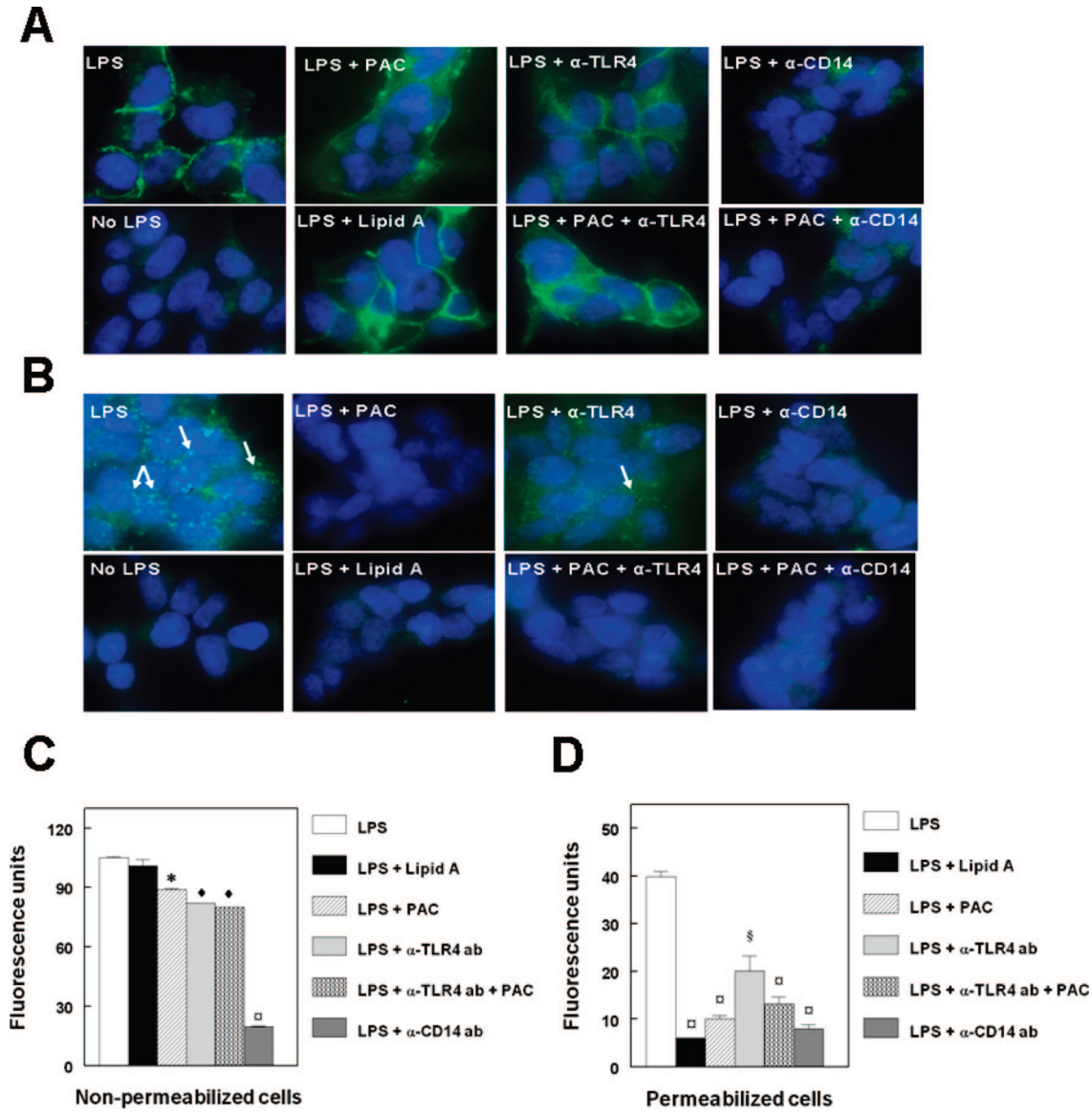

Figure 2. Cranberry PACs slightly reduce membrane binding of LPS and significantly inhibit LPS endocytosis. HEK 293 cells stably expressing CD14 and TLR4/MD2 were incubated with 25 nM LPS and 0.5 μM cranberry PAC for 1.5 h. Cells were either fixed (A) or fixed and permeabilized (B) and incubated with a goat anti-LPS antibody conjugated to fluorescein to visualize LPS. Where indicated, LPS binding was functionally blocked by co-incubation with lipid A or anti-TLR4 and anti-CD14 antibodies. (A) PACs slightly inhibit the binding of LPS to the cell surface. (B) PACs significantly abrogate endocytosis of LPS. The arrows indicate regions of internalized LPS. Nuclei are stained with DAPI. Quantitative analysis of LPS membrane binding and LPS endocytosis are shown in (C) and (D), respectively. Symbols correspond to levels of significance relative to control (determined by Student's *t* test): (*) $p < 0.1$, (♦) $p < 0.05$, (§) $p < 0.01$, (☼) $p < 0.001$.

corresponding increase in the NF-κB response. When the LPS concentration was increased above 2 nM, a slight decrease in the response was noted, due primarily to LPS-induced cytotoxicity (see below). In the presence of 0.5 nM cranberry PACs, a consistent decrease in the NF-κB response was observed across all LPS concentrations. Even when LPS was present at 3 nM (a 6-fold molar excess over PAC), NF-κB activation was not restored to control levels. When cranberry PACs were present at 10 nM, a consistent decrease in the NF-κB response (approximately 50% across all LPS concentrations) was observed relative to the control. Examination of PAC cytotoxicity revealed an IC_{50} for toxicity of 700 nM, with no toxic effects observed at concentrations below 100 nM (Figure 4C). When compared to LPS (IC_{50} for toxicity of ~6 nM), PACs were approximately 100-fold less toxic. In comparison to native LPS, the diphosphoryl form of lipid A did not elicit toxicity at concentrations below 3 μM. Other reports have described the inhibition of LPS-induced production of inflammatory cytokines by PACs. Bodet et al. demonstrated that a PAC-enriched fraction from cranberry juice concentrate inhibited the LPS-induced production of IL-6, IL-8, and prostaglandin E2 in gingival fibroblasts[29] and TNFα (tumor necrosis factor α) and RANTES (regulated on activation normal T-cell expressed and secreted) in macrophages.[30] While this fraction was shown to inhibit the phosphorylation state of intracellular signaling proteins, the exact mechanism of the signaling inhibition was not elucidated. Our findings, however, point to a mechanism of LPS inhibition in which PACs bind directly to and neutralize LPS by blocking its interaction with the receptors TLR4/MD2 and CD14. Further, this interaction with LPS is not readily overcome by excess concentrations of LPS. Studies are currently ongoing in our laboratory to elucidate the precise molecular nature of PAC-LPS interaction.

In this report we have identified PACs from multiple plant sources as a new class of LPS-binding substance. We have demonstrated that PACs possess many of the key attributes required

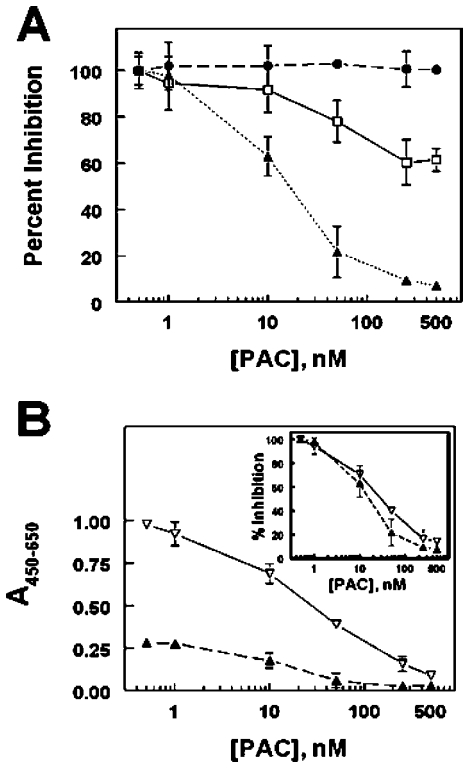

Figure 3. Cranberry PACs inhibit LPS interaction with CD14 and TLR4/MD2 but not with LBP. (A) PACs completely inhibit binding of *E. coli* LPS to immobilized TLR4/MD2 (solid triangles) and partially inhibit binding of LPS-FITC to immobilized CD14 (open squares). No inhibition of LPS:LBP interaction was noted (solid circles). (B) PACs inhibit both the direct and CD14-mediated binding of LPS-FITC to TLR4/MD2. In the presence of 25 nM CD14, the binding of LPS to immobilized TLR4/MD2 is enhanced approximately 4-fold (open triangles) relative to when soluble CD14 is absent (solid triangles). The inset shows both data sets plotted as percent of control. In both instances, the degrees of inhibition to immobilized TLR4/MD2 are comparable. Data are the mean ± standard deviation of two representative experiments.

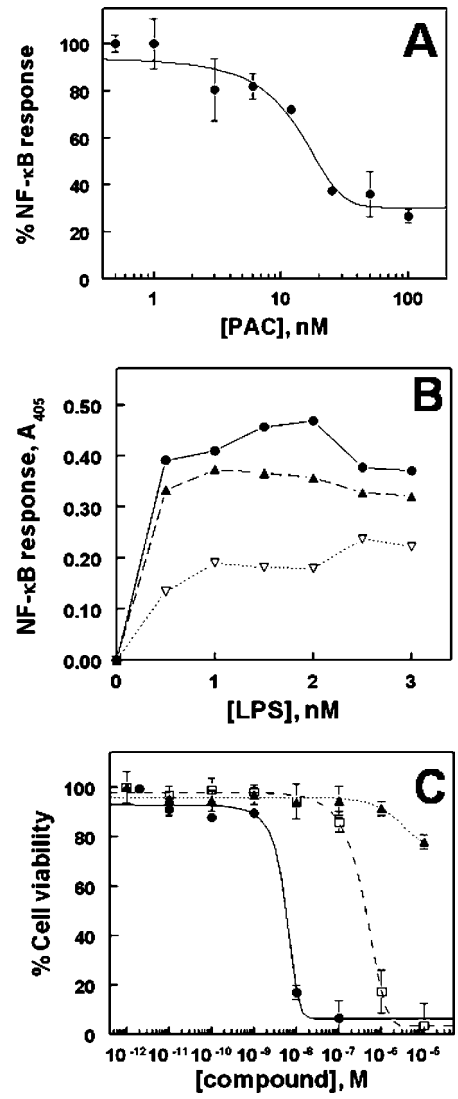

Figure 4. Inhibition of NF-κB activation by and cytotoxicity of PACs in LPS-responsive HEK 293 cells. (A) Cranberry PACs inhibit LPS-induced NF-κB activation in a dose-dependent manner. (B) The inhibitory effect of PACs is not overcome by excess LPS. HEK-CD14-TLR4/MD2 cells were stimulated with LPS at the indicated concentrations in the presence of cranberry PAC at the following concentrations: 0 nM (solid circles), 0.5 nM (solid triangles), or 10 nM (open triangles). (C) PACs are not toxic to LPS-responsive cells over the same concentration range at which they inhibit NF-κB activation, and PACs are ~100-fold less toxic than LPS. Symbols correspond to LPS (solid circles), cranberry PAC (open squares), lipid A (solid triangles).

for their implementation in such analytical applications as the removal and purification of LPS. First, PACs bind LPS with an affinity that is comparable to other well-known LPS-binding compounds (e.g, cationic peptides such as polymyxin B and polyamines). Second, PACs appear to preferentially recognize the conserved lipid A moiety of LPS, a feature that enables PACs to bind LPS from multiple Gram-negative bacterial species. Further, PACs specifically neutralize the endocytosis of LPS by blocking the interaction of LPS with its cognate receptors, TLR4/MD2 and CD14. Abrogation of LPS-receptor interaction effectively inhibits the LPS-induced activation of the transcription factor, NF-κB, and suggests that PACs could also be useful for the *in vivo* treatment of Gram-negative bacterial infections.

Experimental Section

Proanthocyanidins. Dialyzed cranberry juice concentrate (DCC) was produced from Mountain Sun pure unsweetened cranberry juice (100% strength, Celestial Group, Inc.) by dialysis against water [6000 MWCO (molecular weight cut-off) dialysis tubing] and filtration through a 0.2 μm filter. Briefly, PACs (in which nonspecific polyphenols have been removed) were obtained from whole cranberry juice, Welch's 100% red grape juice, or Lipton black tea via purification by hydrophobic adsorption chromatography using a Sephadex LH20 column as described elsewhere.[7,31,32] Whole juice was reduced by rotary evaporation to a minimum volume and resuspended to the original volume in 70% acetone, sonicated for 30 min, and filtered with Whatman #3 filter paper. Resuspension, sonication, and filtration of the insoluble material was repeated twice more, and all liquid was combined. This solution was reduced by rotary evaporation to remove all acetone and resolubilized in 75% EtOH to twice the original volume. Tea was extracted by sonication of one family-sized tea bag in 200 mL of 70% acetone for 20 min (repeated three times). For each preparation, the solutions were combined, reduced by rotary evaporation, and resolubilized in 200 mL of 75% EtOH. This solution was applied to a Sephadex LH20 column in batches equal to the bed volume. Low molecular weight phenolics were removed by elution with ethanol equivalent to 5 times the bed volume. PACs were eluted with acetone and reduced by rotary evaporation to a minimum volume. PACs recovered from whole cranberry juice (Cran PAC) were subsequently fractionated by differential dialysis against H_2O containing 25% EtOH for further characterization. Fractions were collected as those that pass through 2000 MWCO tubing (Spectra/Por; Dial <2K); those that pass through 3500 MWCO tubing (Spectra/Por CE) but are retained by the 2000 MWCO tubing (Dial 2–3K); those that pass through 6000 MWCO

tubing (Spectra/Por MWCO 6–8000) but are retained by the 3500 MWCO tubing (Dial 3–6K); and those that are retained by the 6000 MWCO tubing (Dial >6K). This separation by dialysis is not expected to provide fully isolated components but rather to enrich for the indicated fraction. All materials were dried under an N_2 stream for storage. Purified materials were dissolved in 33% EtOH in H_2O for use in binding experiments. The degree of polymerization of each purified compound was determined by modified vanillin assay combined with acid butanol assay.[31,33] Results of the acid butanol assay are dependent upon the reactivity of the interflavanol bonds. As purified standards are not available, the method provides only an estimate of the degree of polymerization of the materials. The method is, however, valid for determination of variations in fractions from a given species as in the case of the dialyzed materials. PAC concentrations were determined by radial diffusion assay using tannic acid as a standard.[31,34] Analysis of purified materials by thiolysis and HPLC indicated no low molecular weight species remaining after Sephadex LH20 separation.[35–38] On the basis of these analyses, the PACs used in these experiments were considered to be devoid of sugars, acids, and low molecular weight contaminants.

LPS Binding Assays. Polymyxin B (10 μM, conjugated to agarose beads (Sigma)) was incubated with 100 nM LPS-FITC (*E. coli* serotype B5:055, Sigma) in the absence or presence of DCC, nondialyzed Sephadex LH20 PAC, or size-fractionated Sephadex LH20 PAC in a final volume of 250 μL of 0.05 M Tris buffer (pH 8.5). Reactions were stirred for 1 h at 25 °C in the dark. Unbound LPS-FITC was removed by three rounds of centrifugation and washing with 250 μL of 0.05 M Tris buffer, followed by resuspension in 200 μL of nuclease-free H_2O. Serial dilutions of each sample were prepared in nuclease-free H_2O, and the fluorescence was measured by excitation at 495 $\pm$ 2.5 nm and emission at 535 $\pm$ 2.5 nm using a Saphire fluorescence plate reader (Tecan, Durham, NC). Comparable experiments were performed with LPS from *Salmonella*, *Shigella*, and *Pseudomonas* and LPS from mutant strains of *Salmonella Minnesota* (Rc mutant) and *E. coli* EH100 (Ra mutant). The latter two strains contain polysaccharide chains of varying lengths relative to native LPS. Binding experiments were also performed with diphosphoryl lipid A. Conjugation of LPS and lipid A was performed with fluorescein isothiocyanate (Sigma) per the manufacturer's instructions, and the conjugates were subjected to dialysis against PBS to remove unincorporated dye. In all cases, the degree of conjugation was determined by spectroscopy to be approximately 2–3 fluoresceins per mole of labeled species.

Analysis of LPS Membrane Binding and Endocytosis. Human embryonic kidney cells (HEK 293) stably expressing human CD14 and TLR4/MD2 (HEK-CD14-TLR4/MD2; Invivogen) were grown in chambered wells and incubated with 25 nM LPS (*E. coli* serotype O55:B5, Sigma) in the absence or presence of 0.5 μM Sephadex LH20-purified PACs (Dial >6K) for 1.5 h at 37 °C. In control experiments, TLR4 or CD14 was functionally blocked by co-incubation with an anti-TLR4 or anti-CD14 monoclonal antibody (500 nM in binding sites, Abcam, Inc.) or lipid A (Sigma). After incubation, the cells were washed with PBS (10 min) twice and either fixed (with 3.7% paraformaldehyde) to assess LPS membrane binding or fixed and permeabilized (with 0.1% Triton X-100) to determine LPS internalization. After blocking with 1% normal goat serum, membrane-bound or internalized LPS was detected using a goat anti-LPS antibody (O/K serotype-specific, Abcam) conjugated to fluorescein. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). Imaging was performed using an Olympus IX-71 microscope. The relative amounts of membrane-associated or intracellular fluorescence were quantified by image analysis using Image J software (NIH, v. 1.37). Data are reported as the mean channel fluorescence from membrane-associated or internalized LPS and represent the analysis of 10 to 20 cells from each sample (minimum 10 measurements per each cell). Merged images were produced using Photoshop CS2 (ver. 9).

PAC Inhibition of LPS Binding to LBP, CD14, and TLR4/MD2. Human CD14 (Cell Sciences) was adsorbed onto ELISA plates in PBS overnight at 4 °C. Histidine-tagged-human TLR4/MD2 (R&D Systems) or human LPS-binding protein (LBP, Biometec) was captured overnight at 4 °C onto ELISA plates prepared by the passive adsorption of antipolyhistidine monoclonal antibody (R&D Systems). Wells were blocked for 30 min at 37 °C with 1% normal goat serum in PBS. Binding of 5 nM *E. coli* LPS-FITC was performed for 30 min at 37 °C in 1% fetal bovine serum in PBS in the presence or absence of LH20 PAC. Soluble CD14, when present, was at a final concentration of 25 nM. Bound LPS-FITC was detected using a goat antifluorescein-horseradish peroxidase conjugate (Abcam) and tetramethylbenzidine substrate (Kierkegaard and Perry). In the absence of serum, binding of LPS-FITC to CD14 or to TLR4/MD2 was below the detection limit.

Quantification of NF-κB Activation. HEK-CD14-TLR4/MD2 cells were transiently transfected with the NF-κB-inducible reporter plasmid, pNiFty2-SEAP (Invivogen), which encodes secreted embryonic alkaline phosphatase (SEAP) under the control of a 5 $\times$ NF-κB-inducible promoter. Cells were seeded into wells of a 96-well plate (4×10^4 cells/well) and transfected using Effectene reagent (Qiagen) per manufacturer's instructions. After 48 h, the cells were stimulated for 16 h with 2 nM LPS in the presence or absence of cranberry PACs. SEAP activity was measured in tissue culture supernatants using a colorimetric SEAP assay kit (Invivogen) according to the manufacturer's protocol.

Cytotoxicity assays. Cellular toxicity was measured using a colorimetric cell proliferation assay (CellTiter96, Promega). HEK-CD14-TLR4/MD2 cells were seeded into the wells of a 96-well plate (1×10^4 cells/well) and cultured with a dose range of test compounds for 48 h prior to assay according to the manufacturer's instructions.

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Supporting Information Available: Supporting Information providing further evidence of the specific interaction of PACs with LPS and the specific nature of the inhibition of LPS endocytosis by PACs is available free of charge via the Internet at http://pubs.acs.org.

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