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Upregulation of Vitamin D Binding Protein (Gc-Globulin) Binding Sites During Neutrophil Activation from a Latent Reservoir in Azurophil Granules

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Abstract

Vitamin D binding protein (DBP) is a multifunctional plasma transport protein that is also found on the surface of many cell types. Cell surface DBP significantly enhances chemotactic activity of complement (C) peptides C5a and C5a des Arg. However, both DBP binding and C5a chemotaxis enhancement can vary among neutrophil donors. To test if activation during cell purification is responsible for this variability, neutrophils were isolated using both standard and LPS-free protocols. Cells isolated by the LPS-free method had no DBP-enhanced chemotaxis to C5a or DBP binding to plasma membranes. Moreover, neutrophils treated with LPS bound more avidity to immobilized DBP than sham-treated cells. Subcellular fractionation of neutrophils (standard protocol) revealed a heavy plasma membrane (HM) band that contained components of light plasma membranes and all three granules. The HM band possessed most of the DBP binding activity (58%), and activation of cells with ionomycin greatly increased DBP binding to HM. Azurophil granules contained 33% of the total DBP binding sites and there was a highly significant positive correlation ($r = 0.988$) between release of the granule marker myeloperoxidase and DBP binding. These results indicate that fusion of granules with the plasma membrane forms HM that contains DBP binding sites.

Keywords

Complement; Chemotaxis; Inflammation; Vitamin D Binding Protein; Neutrophils; Plasma Membrane

INTRODUCTION

The vitamin D binding protein (DBP), also known as Gc-globulin, is a multifunctional, albumin-like 56 kDa plasma protein that can bind several diverse ligands (White and Cooke, 2000). DBP functions to transport vitamin D sterols and fatty acids, acts as a scavenger protein to clear extracellular G-actin released from necrotic cells, and a deglycosylated form of DBP has been shown to be a macrophage and osteoclast activating factor (White and Cooke, 2000). The chemotactic activity of the complement (C) activation peptides C5a and C5a des Arg also can be enhanced significantly by DBP (Kew and Webster, 1988; Perez et al., 1988),

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an observation independently verified by several different groups (Binder et al., 1999; Metcalf et al., 1991; Petrini et al., 1991; Piquette et al., 1994; Senior et al., 1988; Zwahlen and Roth, 1990). This positive chemotactic cofactor function of DBP (i.e., co-chemotactic activity) is most readily observed using either suboptimal or non-chemotactic concentrations of the C5-derived peptides and is very specific for C5a/C5a des Arg (Kew et al., 1995a; Kew et al., 1995b; Kew and Webster, 1988; Perez et al., 1988). However, DBP by itself lacks chemotactic activity (Kew et al., 1995a; Kew and Webster, 1988). Plasma-derived DBP also binds to the surface of many cell types including neutrophils (DiMartino and Kew, 1999; DiMartino et al., 2001; White and Cooke, 2000). DBP appears to bind with low affinity to multiple cell surface ligands such as chondroitin sulfate proteoglycans (DiMartino and Kew, 1999), megalin (Nykjaer et al., 1999; Nykjaer et al., 2001), cubulin (Nykjaer et al., 2001), CD44 and annexin A2 (McVoy and Kew, 2005). Neutrophils transiently generate co-chemotactic activity for C5a on the cell surface within 15–20 min of DBP binding (Kew et al., 1995a). These cells also utilize membrane-bound elastase to shed the DBP-binding site complex into the extracellular milieu (DiMartino et al., 2001). Both plasma membrane binding and subsequent shedding of DBP are essential in order for the protein to function as a chemotactic cofactor for C5a (DiMartino et al., 2001; Kew et al., 1995a).

The binding of DBP to cells is required for the protein to mediate its numerous functions: a chemotactic cofactor for C5a, a macrophage or osteoclast activating factor, clearance of DBP-actin complexes by the liver and delivery of vitamin D sterols and free fatty acids to cells. Thus, expression of cell surface DBP binding sites is a key factor that limits the functions of this protein. We have noted that the DBP-enhanced neutrophil chemotactic response to C5a can vary considerably among different blood donors, and have speculated that neutrophils may need to be activated in order to bind DBP and show a co-chemotactic response to C5a. The objective of this study was to compare DBP binding and chemotactic enhancement to C5a in neutrophils prepared using a lipopolysaccharide (LPS) free method versus a standard cell isolation protocol. Results clearly show that activated neutrophils bind DBP and display enhanced chemotaxis to C5a. Furthermore, DBP binds to a unique “activated” region of the plasma membrane that contains contents of intracellular granules.

MATERIALS AND METHODS

Reagents

Purified recombinant human C5a, formyl-norleucyl-leucyl-phenylalanine (fNLP), cytochalasin D, monensin, and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO). Vitamin D binding protein (DBP) was purified from human plasma and purchased from Athens Research and Technology (Athens, GA). Lipopolysaccharide (LPS) from *E. coli* 0111:B4 was obtained from Sigma. The protease inhibitors PMSF, 1,10 phenanthroline, E-64, leupeptin and pepstatin A were purchased from Sigma while Pefabloc SC was purchased from Roche Applied Science (Indianapolis, IN). The chemical crosslinker 3,3'-dithiobis (sulfo-succinimidylpropionate) (DTSSP), and microBCA protein measurement kits were purchased from Pierce Chemical Co. (Rockford, IL). Sterile water, HBSS, PBS and 1 M HEPES solution were purchased from Mediatech (Herndon, VA).

Isolation of Human Neutrophils

Neutrophils were isolated from the venous blood of healthy, medication-free, paid volunteers who gave informed consent. Leukocyte-rich concentrates (Leukopaks) also were purchased from the New York Blood Center (Long Island Blood Services, Melville, NY). Two procedures were used to isolate neutrophils, a standard 3-step procedure (both freshly drawn blood and Leukopaks) and an LPS-free method (used on freshly drawn blood only). The standard three-step isolation procedure has been described previously (Kew et al., 1992). All materials used

in this procedure were sterile but not certified LPS-free. Briefly, heparinized blood (10 U/ml) was treated with an equal volume of 3% dextran T-500 (Amersham BioSciences, Piscataway, NJ), prepared in PBS, for 30 min to sediment the RBCs. After washing the cells in PBS, the leukocyte-rich pellet was centrifuged through a Lymphoprep (Accurate Scientific, Westbury, NY) gradient (density = 1.077 g/ml) at 400 X g for 30 min at 22°C to separate the mononuclear leukocytes from the neutrophils. The neutrophil-rich pellet was resuspended in PBS and exposed to hypotonic conditions (75 mosM) for 60 s to lyse any contaminating RBCs. Neutrophils were then washed twice in PBS, counted, and placed on ice. Isolation of cells using an LPS-free protocol was similar to the standard protocol with three exceptions. First, blood was collected in tubes containing 7% EDTA; second, all material, including glassware, was LPS-free; third, the step for hypotonic lysis of RBC was omitted. Both protocols yielded similar cell preparations that contained 98% neutrophils, 1 % eosinophils, and 1% mononuclear cells, and were 99% viable by trypan blue dye exclusion.

Chemotaxis Assay

Cell movement was quantitated using a 48 well microchemotaxis chamber (Neuroprobe, Cabin John, MD) and 5.0 µm pore size cellulose nitrate filters (purchased from Neuroprobe) as previously described (Kew et al., 1995a). Cell movement was quantitated microscopically by measuring the distance in microns (µm) that the leading front of cells had migrated into the filter according to the method described by Zigmond and Hirsch (Zigmond and Hirsch, 1973). In each experiment, five fields per duplicate filter were measured at 400 X magnification. The value of the background controls for random cell movement (cells responding to buffer) has been subtracted in all cases so that the data are presented as net movement in µm.

Neutrophil-DBP Binding Measured by Biacore Surface Plasmon Resonance

Binding to immobilized DBP was measured by surface plasmon resonance using a Biacore 2000 (Biacore, Piscataway, NJ). Purified DBP in HBSS (5 µM) was coupled to Biacore CM5 sensor chip. Coupling was verified using 5 µg/ml affinity-purified anti-DBP. Neutrophils (10⁶/ml) in HBSS were injected into the flow cell and allowed to interact with the immobilized DBP and the binding analyzed in real-time. Data is expressed as response units of the molecular interaction on the sensor chip.

Disruption and Subcellular Fractionation of Neutrophils

The cell disruption and subcellular fractionation protocols were originally described by Borregaard et al. (Borregaard et al., 1983). Briefly, purified neutrophils were suspended at 75–100 x 10⁶ cells/ml in ice cold disruption buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM HEPES, 1 mM ATP, pH 7.4, containing 1 mM PMSF, 2 mM 1,10 phenanthroline, 100 µM E-64 and 25 µM Pepstatin A) then added to a prechilled nitrogen cavitation bomb (Parr Instrument Company, Moline, IL). The cells were equilibrated to 350 psi with constant stirring for 20 minutes and then collected in a dropwise fashion into 125 mM EGTA, pH 7.4 to achieve a final concentration of 1.25 mM EGTA. Nuclei and unbroken cells were removed by centrifugation at 800 x g for 10 minutes at 4°C. Prechilled (4°C) Percoll gradients (Amersham Biosciences) were prepared by progressively underlaying 2.2 ml of each increasing density (1.05, 1.09 and 1.12 g/ml) in a 14 x 89 mm Ultra-Clear centrifuge tube (Beckman Instruments, Palo Alto, CA). In other experiments, a fourth layer of 1.07 g/ml Percoll was added. The supernatant from the nitrogen cavitation (approximately 3 ml) was layered on top of the Percoll gradient and centrifuged at 50,000 x g for 50 minutes in a prechilled SW41 swinging bucket rotor (Beckman). Fractions were collected by aspiration using sterile, glass Pasteur pipettes. Percoll was removed from the fractions by centrifugation at 100,000 x g for 60 min at 4°C. The purified neutrophil subcellular fractions were resuspended in equal volumes of HBSS, the

total protein content then was measured using the micro BCA assay and the purity of each subcellular fraction was assessed using marker assays for plasma membranes and each granule population (Borreagaard et al., 1983). Purity of the plasma membrane and azurophil granule preparations were evaluated using assays for alkaline phosphatase and myeloperoxidase respectively. The purity of gelatinase granules was assessed using gelatin zymography. A western blot for lactoferrin was used to evaluate the purity of specific granules.

Assay for Alkaline Phosphatase

Alkaline phosphatase and latent alkaline phosphatase were assayed using 1 mg/ml *p*-nitrophenylphosphate as a substrate in 50 mM sodium barbital (pH 10.5) containing 1 mM MgCl₂. For latent alkaline phosphatase, 0.2 % Triton X-100 (TX-100) was included in the buffer to permeabilize the membranes. The samples were incubated at 37°C for 30 minutes, and the reaction was terminated by the addition of 150 µl ice cold buffer without substrate. Absorbance was immediately read at 405 nm. Latent alkaline phosphatase was determined by subtracting the absorbance of the sample in the absence of TX-100 from that obtained in the presence of TX-100.

Assay for Myeloperoxidase

Myeloperoxidase (MPO) was assayed using DMB (*o*-dianisidine) as a substrate that yields an orange color when it reacts with oxygen radicals. The material from subcellular fractions was disrupted using TX-100 and then were diluted in 0.2 M Na₂PO₄ (pH 6.2) to bring the final concentration of TX-100 to less than 0.02%. Supernatants from intact cell binding assays were tested for MPO activity without alterations. The reaction buffer included 0.2 M Na₂PO₄ (pH 6.2), HBSS-0.25% BSA, 0.05% H₂O₂ and 1.25 mg/ml DMB. Samples were mixed, and incubated for 10 minutes at room temperature. The reaction was terminated by the addition of 1% NaN₃ and the absorbance was read at 450 nm.

Gelatin Zymography

Gelatinase was assayed using gelatin zymography. Non-reduced samples were prepared for SDS-PAGE without boiling. Substrate gels were prepared by co-polymerizing acrylamide with 0.028% gelatin (Sigma). Following electrophoresis (4 W/gel maximum), SDS was eliminated from the gels by washing in 2.5% TX-100. Next, the gels were transferred to assay buffer (40 mM Tris, 0.2 M NaCl, 10 mM CaCl₂, pH 7.5) and incubated 4 hours at 37°C. Gels were stained in Coomassie blue overnight and destained for approximately one hour. Gelatinase activity was identifiable as clear zones of gelatin degradation and bands were quantitated by densitometry.

Western Blot for Lactoferrin

Samples separated by SDS-PAGE were transferred from the gel to 0.45 µm pore size PVDF membrane (Millipore, Bedford, MA) using a Bio-Rad semi-dry blotting apparatus (Transblot SD) at 14 V for 15 minutes at room temperature. The blot was then incubated for 45 minutes at room temperature in a blocking solution of PBS containing 5% nonfat dry milk. After a brief wash in PBS containing 0.025% NaN₃ to remove blocking solution, the blot was incubated 18 hours with rabbit anti-human lactoferrin (Bioscience International) at 7.5 µg/ml in PBS containing 0.025% NaN₃ and 0.1% Tween 20 (PBS-Tween). After washing twice in PBS-Tween, the blot was incubated for 45 minutes with affinity purified goat anti-rabbit IgG F (ab')₂ conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) at 0.5 µg/ml in PBS-Tween. After washing twice in PBS-Tween, the blots were developed using an enhanced chemiluminescence kit (ECL, Amersham, Arlington Heights, IL) and the lactoferrin band quantitated by densitometry.

Quantitative DBP Binding Assay

Purified DBP was radioiodinated as described previously in detail (DiMartino and Kew, 1999). DBP (200 μg) was radioiodinated using one Iodobead (Pierce Chemical Co.) and 1 mCi of $\text{Na-}^{125}\text{I}$ (PerkinElmer Life Sciences, Shelton, CT) for 5 min. Briefly, neutrophils (10^7 cells/sample) or subcellular fractions (10 $\mu\text{g}/\text{sample}$) were incubated with 100 nM ^{125}I -DBP in HBSS containing 0.1% BSA (assay buffer) in a total volume of 100 μl . Samples were incubated for 30 min at 37°C. After the incubation period, the samples were applied to a Millipore vacuum filtration manifold to separate free from bound radiolabeled ligand. Samples were separated using 25 mm, circular Durapore type VV filters with 0.1 μm pore size (Millipore). The filters were presoaked with 1% BSA (in HBSS) to block nonspecific binding of protein. After vacuum separation, the material bound to the filters was washed four times with 1 ml of ice-cold assay buffer, then the filters counted in a gamma counter for the amount of bound ^{125}I -DBP. All samples were assayed in triplicate or quadruplicate. For each concentration of radiolabeled DBP, a buffer control was included that contained no cells or membranes. Radioactivity that bound to filters under these conditions was considered the background value and was subtracted from the appropriate samples.

Data Analysis and Statistics

A minimum of 3 experiments was performed for each assay. Results of several experiments were analyzed for significant differences using either the T-test or linear regression with the statistical software program InStat (GraphPad Software, San Diego, CA).

RESULTS

The DBP-enhanced neutrophil chemotactic response to C5a can be variable, and it has been speculated that neutrophils need to be activated to some extent in order to bind DBP and show a co-chemotactic response to C5a (McVoy and Kew, 2005). This may explain why it often takes 15 to 20 min after purified DBP is added to neutrophils before C5a co-chemotactic activity is detected in vitro (Kew et al., 1995a). Indeed, we have observed a dramatic difference in both DBP binding and C5a chemotactic enhancement in cells isolated from blood using minimal manipulation and the most stringent LPS-free conditions. Figure 1A demonstrates the significant differences in DBP binding and C5a chemotaxis enhancement between neutrophils isolated using a standard 3-step protocol versus an LPS-free method. There was essentially no DBP binding to isolated plasma membrane fractions and no DBP-enhanced chemotaxis to C5a in neutrophils isolated by the LPS-free protocol, whereas cells purified by a standard method displayed significantly greater ($p < 0.001$) DBP binding and enhanced chemotaxis (Fig. 1A). To verify that LPS-treated neutrophils bind DBP more avidity than untreated cells, purified neutrophils (isolated using the standard protocol) were treated with 2 $\mu\text{g}/\text{ml}$ LPS for 30 min, washed 2x, and then allowed to interact with purified DBP immobilized to Biacore CM5 chip and binding monitored in real-time by surface plasmon resonance (Fig. 1B). LPS-treated cells clearly demonstrate enhanced binding to DBP versus sham-treated control cells. Neither sham nor LPS-treated neutrophils bound to a control chip containing BSA (data not shown). These results confirm that neutrophils need to be activated to bind DBP and display enhanced chemotaxis to C5a.

A distinct difference was noted in the appearance of the plasma membrane fraction between neutrophils isolated using the LPS-free versus the standard protocol. Subcellular fractionation of disrupted cells on 3-layer discontinuous Percoll gradients (1.05, 1.09, 1.12 g/ml) yields a plasma membrane enriched band on top of the 1.05 g/ml Percoll (Borregaard et al., 1983). Neutrophils purified using the LPS-free method produced a clearly-defined plasma membrane band, while cells isolated by the standard protocol had high-density material trailing from 1.05 to 1.09 g/ml (data not shown). This material was even more prominent if neutrophils were

obtained from a Red Cross Leukopak. To define this plasma membrane material a fourth Percoll density layer of 1.07 g/ml was added to the discontinuous gradients. Figure 2A shows that a distinct band forms on top of the 1.07 g/ml layer that we have designated as heavy plasma membranes (PM-heavy) to distinguish from the typical light plasma membrane (PM-light) band on top of the 1.05 g/ml Percoll. Three neutrophil cytoplasmic granule fractions were also isolated (Fig. 2A): gelatinase granules (on top of 1.09 g/ml), specific granules (on top of 1.12 g/ml), azurophil granules (beneath 1.12 g/ml). The purity of each fraction was assessed by measuring alkaline phosphatase for plasma membranes, gelatinase for gelatinase granules, lactoferrin for specific granules, myeloperoxidase (MPO) for azurophil granules (Fig. 2B). Heavy membranes contained 25% of the alkaline phosphatase, while light plasma membranes contained the remainder of this enzyme activity (Fig. 2B). This is consistent with the concept that heavy membranes are derived from plasma membranes. Surprisingly, approximately 21% of total gelatinase, 18% of total lactoferrin and 22% of the total MPO activity also was found in the heavy plasma membranes (Fig. 2B). The heterogeneous nature of the heavy membranes was confirmed by examining each fraction by transmission electron microscopy where the heavy membranes appeared to be a mixture of the other four fractions (data not shown). These results clearly demonstrate that the heavy membrane fraction contains markers of all granules and the plasma membrane and may represent an activated region of the plasma membrane.

The capacity of each subcellular fraction to bind radioiodinated DBP, both intact and fractions subjected to eight freeze/thaw cycles to disrupt granules, was determined next (Figure 3A). The majority of DBP binding activity in the intact samples (78%) was found in the heavy membrane fraction while very little radioactivity was detected in the granule fractions (Fig. 3A). Following the freeze/thaw disruption protocol, DBP binding increased in all fractions, but most notably, 90% of the binding activity was found in the heavy membrane and azurophil granule fractions, suggesting that fusion of azurophil granules with the cell surface may generate heavy membranes. To test this premise, neutrophils were pretreated with the calcium ionophore ionomycin (1 μ M for 30 min) to induce exocytosis of granules, this treatment resulted in a 4-fold increase in DBP binding to the heavy membrane fraction (Fig 3B). These results indicate that the heavy membrane DBP binding site is derived from azurophil granules and can be increased by cell activation.

It is possible that the heavy plasma membrane fraction may be an artifact of the nitrogen cavitation and subcellular fractionation protocol. To confirm that a binding site for DBP is located on heavy membranes in viable, intact cells, neutrophils were incubated with radioiodinated DBP in the presence or absence of the water-soluble crosslinker, DTSSP, and then were disrupted and separated into subcellular fractions (Figure 4). Almost all the radioactivity (98%) remained associated with the plasma membrane fractions, confirming that DBP is not internalized as we have reported previously (DiMartino and Kew, 1999). Cells incubated in buffer alone had approximately 75% of 125 I-DBP associated with the heavy plasma membrane fraction. Cross-linking using DTSSP increased the amount of DBP associated with the heavy membranes by 7-fold (Fig. 4). These results suggest that heavy membrane DBP binding site is present in intact cells and is not an artifact of subcellular fractionation.

Finally, the results of Figure 3 indicate that the DBP binding site is derived from azurophil granules fusing with the cell surface to form the heavy plasma membrane fraction. Accordingly, there should be a correlation between DBP binding and azurophil granule release in neutrophils. Various agents known to stimulate neutrophils were used to correlate the binding of DBP to intact cells with the release of the azurophil granule marker myeloperoxidase (MPO) (Figure 5). Treatment of neutrophils with the sodium ionophore monensin (shown to cause a selective release of azurophil granules), phorbol myristate acetate (PMA) or cytochalasin D/formyl peptide (releases all granules) resulted in an increase in both the uptake of DBP and release of

MPO (when compared to an HBSS control). In contrast, incubation of neutrophils on ice (1° C), or in buffer that lacked Ca²⁺ and Mg²⁺ (PBS), resulted in a decrease in both parameters (Fig. 5). Correlation of DBP binding with MPO release generated a highly significant positive correlation coefficient value of 0.988 (Fig. 5). There was no correlation between the uptake of DBP and the release of specific or gelatinase granule markers (data not shown). These results show that there is a highly significant positive correlation between cell surface DBP binding and MPO release, confirming that a reservoir of DBP binding sites is contained in azurophil granules.

DISCUSSION

The results presented in this paper help to clarify how DBP interacts with neutrophils and mediate enhanced chemotaxis to C5a. The objective of this study was to ascertain if neutrophil activation correlates with an increase in DBP binding and chemotactic enhancement to C5a. This question arose from observations that both parameters are variable among neutrophils obtained from healthy human blood donors, even in cells derived from the same individual but on different days. A reasonable explanation for this inconsistency was a differing degree of neutrophil activation among the cell preparations. Indeed, the results demonstrate clearly that quiescent neutrophils do not bind DBP nor display enhanced chemotaxis to C5a. Physiologically, this would make sense because pro-inflammatory functions are not meant to function in the blood and are tightly regulated until the cells exit the vasculature. Thus, DBP binding sites are latent while neutrophils circulate in blood bathed in plasma containing a high concentration of DBP (6-7 μM).

A second major finding of this study is the identification of a heavy plasma membrane fraction that is comprised of constituents derived from light plasma membranes and all three granule populations. The heavy membrane fraction contains the majority of the DBP binding sites and may represent “activated” plasma membranes. Previously, several studies have reported heavy and light plasma membranes isolated from neutrophils using a continuous sucrose density gradient (Jesaitis et al., 1988; Jesaitis et al., 1993; Jesaitis et al., 1989). Jesaitis et al. demonstrated that the heavy plasma membrane fraction contains activated formyl peptide receptors interacting with the cytoskeletal proteins fodrin and actin, while the majority of the alkaline phosphatase was localized to the light membrane fraction (Jesaitis et al., 1988; Jesaitis et al., 1993; Jesaitis et al., 1989). Although it is not clear whether the heavy membranes described in this paper are equivalent to those in the previous reports (since fractionation on continuous sucrose gradients is different than the discontinuous Percoll gradients used herein), there are some similarities (Jesaitis et al., 1988; Jesaitis et al., 1993; Jesaitis et al., 1989). First, heavy membranes were depleted in alkaline phosphatase compared to the light membranes, and heavy membranes clearly contained activated receptors. Furthermore, this group has also shown that ligand activation shifts the formyl peptide receptor from a detergent soluble to an insoluble fraction in neutrophils (Jesaitis and Klotz, 1993). Previously, we have shown that the neutrophil DBP binding site is located in the detergent insoluble fraction (DiMartino and Kew, 1999). More recently, a proteomic analysis of high-density detergent resistant plasma membranes (DRMs) from neutrophils revealed that this fraction represents a cytoskeleton-associated subset of DRMs (lipid rafts) containing signaling molecules (Nebl et al., 2002). Thus, there is considerable evidence that activated neutrophils have a high-density or heavy region of the plasma membrane that is associated with the cytoskeleton and may be responsible for transmitting extracellular signals. However, it is not clear if the previously reported heavy membranes contain contents of gelatinase, specific and azurophil granules as we have reported herein.

The third major finding of this paper is that azurophil granules are a latent reservoir of DBP binding sites, and their fusion with the plasma membranes greatly increases the neutrophils

capacity to bind DBP. This was an unexpected finding since the azurophil granules are known to contain the bulk of the cytotoxic anti-microbial compounds of the neutrophil, whereas internal stores of receptors generally are contained in the other granule fractions (Faurischou and Borregaard, 2003). In addition, azurophil granules are the last granule population to be mobilized and fuse with the plasma membrane during phagocytosis and/or cell activation (Faurischou and Borregaard, 2003). Azurophil granules also are the storage compartment for elastase (Faurischou and Borregaard, 2003), and we have demonstrated that this protease cleaves the DBP binding site (but not DBP) and sheds it into the extracellular milieu (DiMartino et al., 2001). Inhibition of elastase causes large amounts of DBP to accumulate on the cell surface (a 5-fold increase after 60 min) and also inhibits C5a chemotactic cofactor activity (DiMartino et al., 2001). Thus, elastase-mediated shedding may be a mechanism to terminate the DBP binding/signaling complex. Interestingly, we have previously demonstrated that normal human neutrophils contain small amounts of DBP in specific granules (1.5 ng/10⁶ cells) that may be used during inflammation to enhance C5a chemotaxis and/or scavenge actin (Kew et al., 1993). DBP localized to specific granules also would compartmentalize the protein from its binding sites (plasma membrane, azurophil granule) and keep them separate until the cell is activated. Although the data is quite convincing that azurophil granules contain a DBP binding site, the precise reason it is located in this storage compartment is puzzling. Furthermore, it is not clear if the binding site is a granule membrane component or a matrix molecule bound to the membrane. These questions will require further investigation.

Several molecules have been identified as potential DBP binding sites. Our lab has demonstrated that a chondroitin sulfate proteoglycan (CSPG) binds DBP on neutrophil plasma membranes (DiMartino and Kew, 1999). Moreover, we have subsequently identified the cell surface CSPG CD44, as well as annexin A2, as DBP binding sites on both neutrophils and U937 cells (McVoy and Kew, 2005). Another lab has reported that DBP binds with low affinity to the multi-ligand clearance receptors megalin and cubulin on proximal tubule cells in the kidney (Nykjaer et al., 1999; Nykjaer et al., 2001). Therefore, the binding site in azurophil granules may be one of several DBP ligands in neutrophils, each perhaps binding with a different avidity. We have speculated that the formation of the neutrophil DBP binding site complex is a sequential, multi-step process involving several cell surface receptors and enzymes (McVoy and Kew, 2005). This may explain why it takes at least 15 to 20 min after DBP is added to cells before co-chemotactic activity is detected (Kew et al., 1995a). Previous binding studies in neutrophils support the concept that DBP binds to different molecules at different stages during neutrophil activation. The initial binding to cells is relatively weak but once it has reached a steady-state plateau between binding and shedding (30 min at 37°C) DBP is bound tightly to the detergent insoluble fraction of the plasma membrane and can only be dissociated by harsh conditions (1% SDS or 0.1 M carbonate, pH 11) (DiMartino and Kew, 1999; Kew et al., 1995a). This would suggest that at least one of the DBP ligands binds with high affinity. A well-known high affinity ligand for DBP is the monomeric form of actin (G-actin), and plasma DBP serves as an extracellular scavenger for actin released from necrotic tissue (White and Cooke, 2000). Actin is the most prevalent intracellular protein, however, several investigators have reported actin on the extracellular face of the plasma membrane in lymphocytes and endothelial cells (Dudani et al., 2005; Petrini et al., 1983; Petrini et al., 1985; Wang et al., 2006; Wang et al., 2004). Reports have also shown that other cytoskeletal proteins are expressed on the cell surface (Moisan and Girard, 2006). Furthermore, a recent proteomic analysis of human neutrophil granules has revealed the presence of actin and other cytoskeletal proteins in all three granule populations (Lominadze et al., 2005). Thus, it is conceivable that the tight DBP-membrane binding we have observed is due to the interaction of DBP with cell surface actin. Indeed, we have observed DBP bound to actin on the plasma membrane of both neutrophils and the myeloid cell line U937 (L.A. McVoy and R.R. Kew, manuscript in preparation). The reason that actin is expressed on the extracellular face of the plasma membrane is not clear but may reflect either cell activation or a pre-apoptotic state.

The cell surface DBP binding site is an essential link in DBP physiology since the binding of this protein to cells is required to mediate its numerous functions. However, the plasma membrane DBP ligands are not completely characterized and the process by which the protein interacts with cells is poorly understood. Moreover, it is a distinct possibility that different cell types possess different cell surface ligands for DBP. The characterization of individual DBP binding ligands using traditional biochemical approaches has been limited. It appears that DBP binds to multiple ligands, perhaps in a sequential order, and generally with low avidity, making the task of identifying each ligand exceedingly difficult. Nevertheless, this report has clarified how DBP interacts with neutrophils and may provide a method to dissect the different DBP ligands in this cell type.

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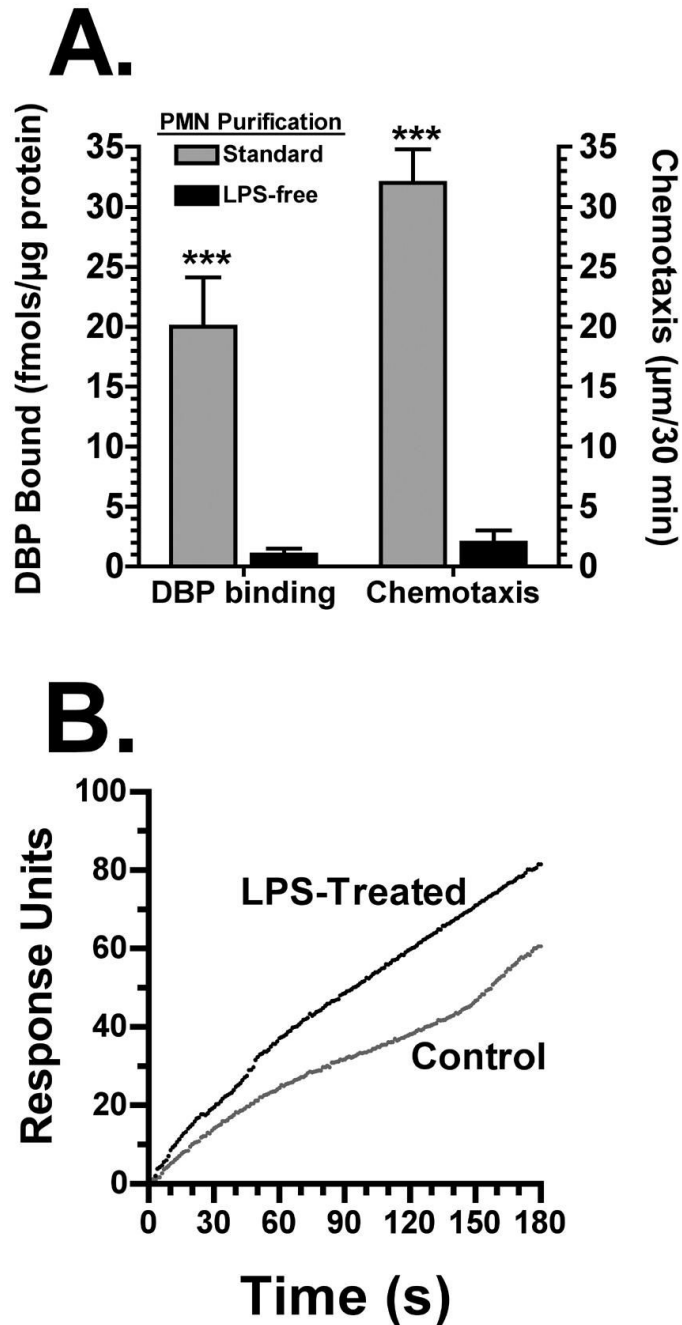


Figure 1. Enhanced C5a chemotaxis and DBP binding in activated neutrophils

Panel A: Neutrophils isolated by either the standard or LPS-free protocol were evaluated for chemotaxis and DBP binding. Chemotaxis to 10 pM purified C5a + 50 nM purified DBP was measured using a filter-based assay. Binding of radioiodinated DBP to plasma membrane fractions was measured using 10 μg of membrane protein incubated in HBSS-0.1% BSA with 100 nM ^{125}I -DBP for 30 minutes at 37°C. Bound label was separated from free using a vacuum filtration unit and filters were counted in a gamma counter. For both assays (n = 3) asterisks indicated that the sample is significantly greater (p<0.001) than the corresponding LPS-free sample. **Panel B:** Purified DBP (5 μM) in HBSS was coupled to a Biacore CM5 sensor chip. Cells were pretreated with 2 μg/ml LPS for 30 min, or sham-treated, then washed twice.

Neutrophils (10^6 /ml) in HBSS were injected into the flow cell and allowed to interact with the immobilized DBP. Data is expressed as response units of the molecular interaction on the sensor chip.

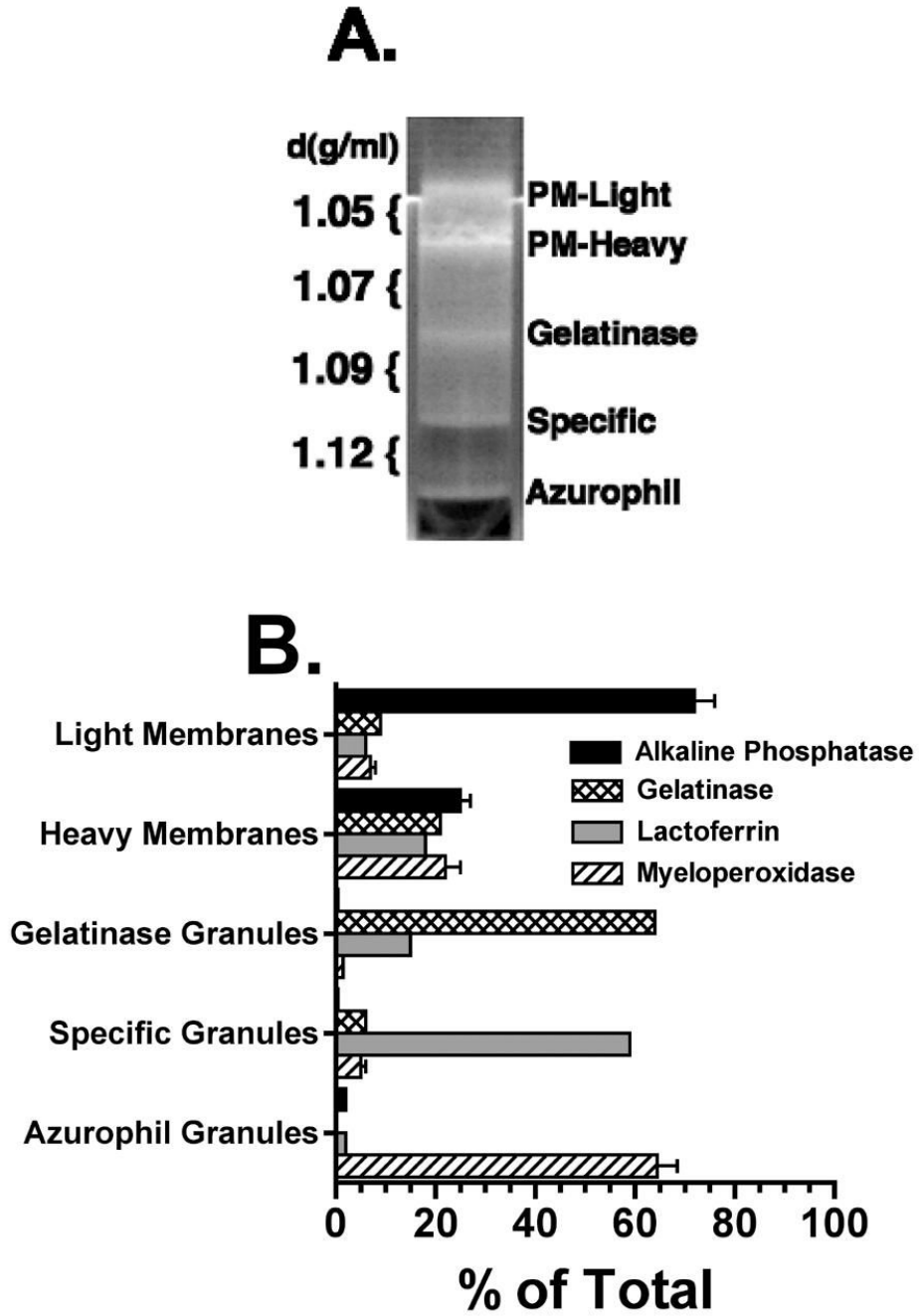


Figure 2. Subcellular fractionation of neutrophils on discontinuous Percoll gradients
Panel A: Purified neutrophils were disrupted at 100×10^6 cells/ml in disruption buffer and then were layered on top of a prechilled four-layer Percoll gradient (see METHODS). The photo shows the gradient after ultracentrifugation. Five distinct fractions are noted. **Panel B:** The purity of each fraction was evaluated using assays for alkaline phosphatase, gelatinase, lactoferrin and myeloperoxidase. Data is reported as the % of total neutrophil activity (combined 5 fractions = 100% for each marker).

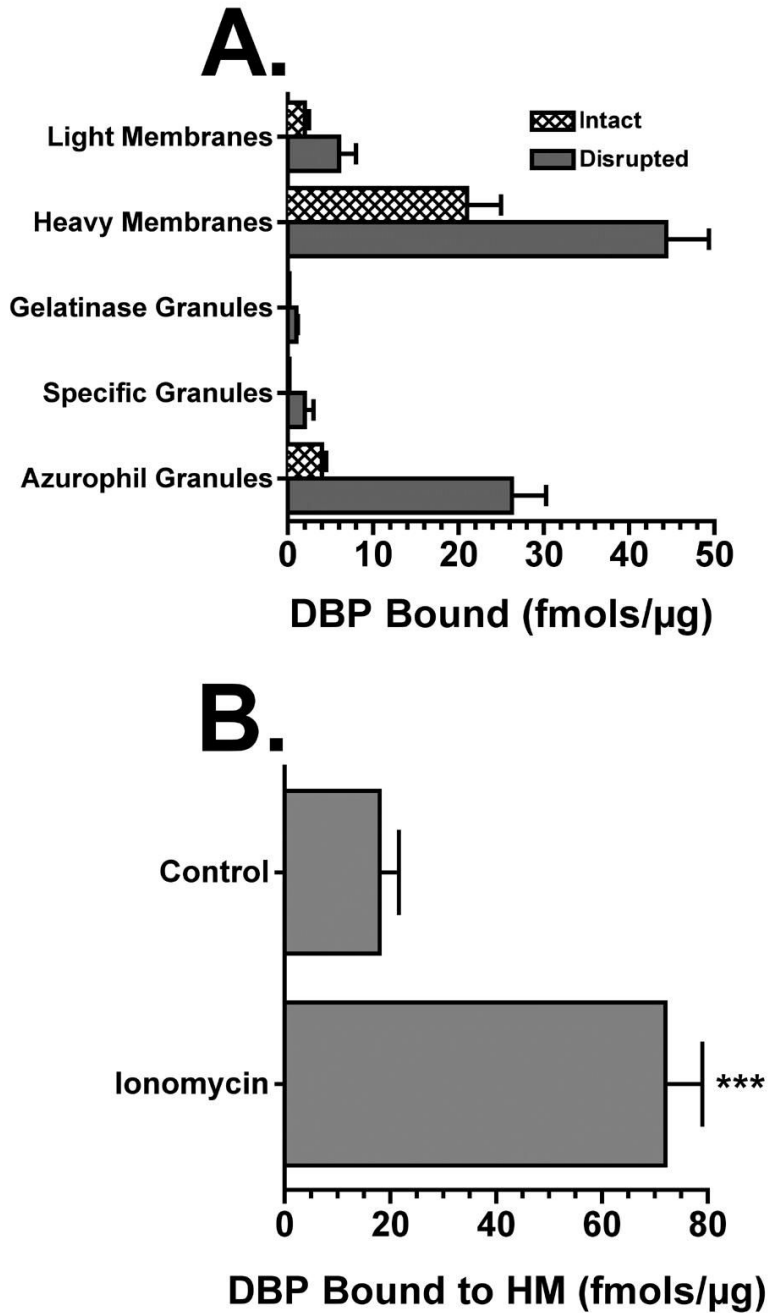


Figure 3. DBP binds to isolated heavy plasma membranes

Panel A. Ten μg of protein from each fraction were incubated in HBSS-0.1% BSA with 100 nM ^{125}I -DBP for 30 minutes at 37°C . In addition, membrane and granule fractions (10 μg total protein) were disrupted using eight cycles of rapid freezing (-80°C) and thawing (in the presence of 1 mM PMSF), these fractions also were incubated in HBSS-0.1% BSA containing 100 nM ^{125}I -DBP for 30 minutes at 37°C . The samples then were filtered and washed as described in METHODS. Filters were counted for radioactivity and blank values were subtracted from each sample value. Data are expressed as fmols DBP bound per μg of protein from each fraction. The results represent the mean \pm SEM of 7 to 12 separate experiments using isolated fractions from different donors. **Panel B.** Neutrophils were either sham-treated or

treated with 1 μM ionomycin for 30 min at 22°C and then were subjected to disruption and subcellular fractionation. Heavy membrane fractions (10 μg) from both sham and ionomycin-treated cells were incubated in HBSS-0.1% BSA (pH 7.4) with 100 nM ^{125}I -DBP for 30 minutes at 37°C. The samples then were filtered and washed as described in METHODS. Data are expressed as fmols DBP bound per μg of HM protein. The results represent the mean \pm SEM of 3 experiments. Asterisk indicates that value is significantly greater ($p < 0.001$) than all other samples.

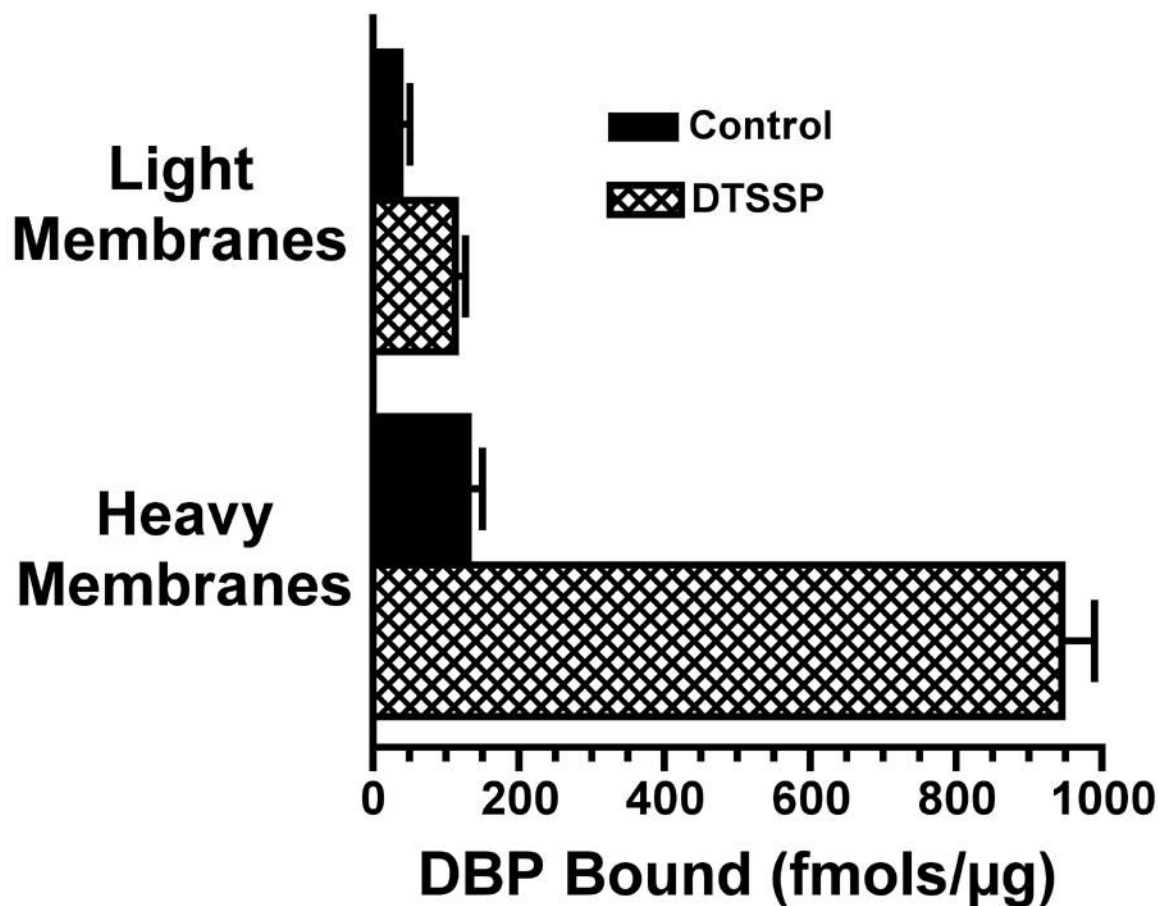


Figure 4. Subcellular fractionation of neutrophils after the uptake of radioiodinated DBP
Purified neutrophils first were incubated in HBSS with 100 nM ^{125}I -DBP for 30 minutes at 37°C. One sample was chilled on an ice slurry, washed with ice cold HBSS and resuspended in disruption buffer at 75×10^6 cells/ml. After disruption in a nitrogen cavitation bomb, the cavitate was saved on ice. Meanwhile, the other sample was chilled on an ice slurry and incubated for 30 minutes on ice in the presence of 1 mM DTSSP. The crosslinking reaction was terminated by adding 1 M Tris, pH 8.0 to a final concentration of 10 mM and incubating for 15 minutes on ice. The cells then were washed and resuspended in ice cold disruption buffer for nitrogen cavitation. Both cavitates then were separated into subcellular fractions on four-layer Percoll gradients. The light and heavy plasma membrane fractions were isolated, washed in cold disruption buffer, and finally counted for radioactivity. Data are expressed as fmols of DBP associated with each fraction.

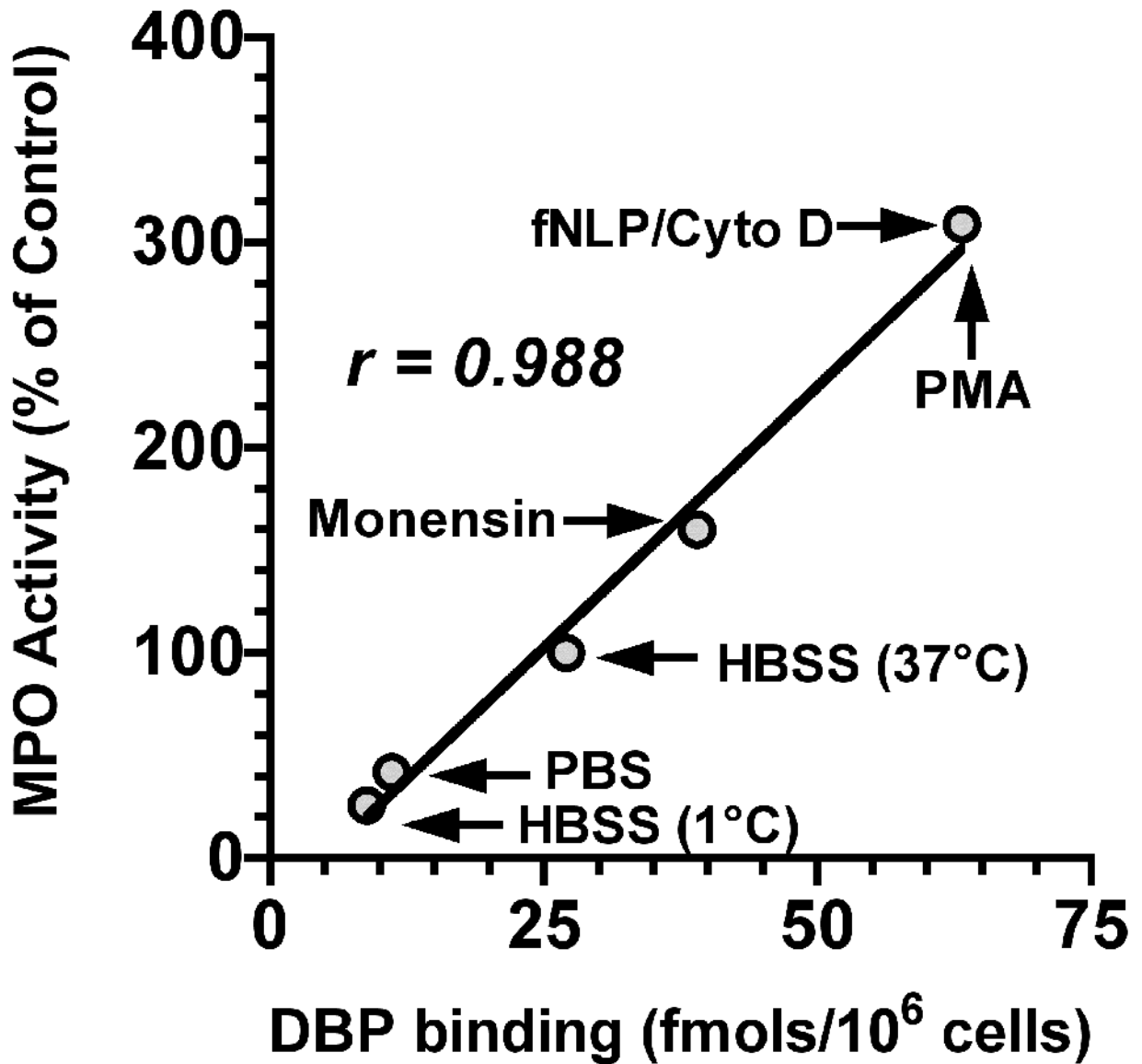


Figure 5. Neutrophil DBP binding correlates with the release of MPO

Purified neutrophils were incubated in one of the following conditions: (1) PBS at 37°C, (2) HBSS on ice (1°C), (3) HBSS at 37°C, (4) HBSS containing 5 nM PMA at 37°C, (5) HBSS containing 5 mg/ml cytochalasin D and 500 nM fNLP at 37°C. The sample containing cytochalasin D was pretreated for 15 minutes at 37°C before the fNLP was added. All samples contained 0.1% BSA and 100 nM ¹²⁵I-DBP and were incubated 30 minutes at 37°C or 1°C as indicated. Following incubation, samples were chilled using an ice slurry for 5 minutes and centrifuged to pellet the cells. The supernatants were collected and assayed for MPO activity. Cell pellets were washed twice in ice cold buffer and counted for radioactivity. The MPO activity is expressed as percent of the MPO activity found in the HBSS at 37°C sample. The binding of DBP is expressed as fmols DBP bound per million neutrophils. The data points of fNLP/cyto D and PMA treated cells are identical. Data represent the mean of 2 to 5 separate experiments performed in triplicate using neutrophils from different donors.