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Characterization of the Lipopolysaccharide Binding Site on the Plasma Protein High Molecular Weight Kininogen

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By
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DEDICATION

I would like to dedicate this thesis to my parents, Terry and Dianna Perkins. Thank you for being supportive of my desire to join the Sally McDonnell Barksdale Honors College, to conduct research and for always stressing the importance of education. I would not be where I am today without you and all that you have done for me.

ABSTRACT

RYAN C. PERKINS: Characterization of the Lipopolysaccharide Binding Site on the Plasma Protein High Molecular Weight Kininogen

Bradykinin (BK) participates in the control of local blood flow and blood pressure by causing vascular hyperemia and vascular inflammation. The Bradykinin generating pathway of human plasma consists of prolylcarboxypeptidase (PRCP), prekallikrein (PK), and high molecular weight kininogen (HK). When the complex of HK-PK assembles on endothelial cells or an artificial negatively charged surface, PK is immediately converted to kallikrein by the enzyme PRCP. The formed kallikrein then autodigests HK to liberate BK. The BK that is formed then plays an important role in vascular permeability by stimulating constitutive Bradykinin B2 and inducible B1 receptors to release prostacyclin and nitric oxide. Thus, the activity of PRCP leads to vasodilation. In inflamed tissues, tissue injury or hypotensive bacteremia, BK has been shown to accumulate in the blood or plasma of patients. Since LPS possesses a negatively charged surface and the ability to stimulate an inflammatory response, the interaction of LPS with the proteins of the Bradykinin generating pathway assessed and characterized. These studies showed that the binding of LPS to HK was time dependent, dose dependent, saturable and reversible. The binding of LPS to HK was shown to occur via the positively charged amino acid residues of domain 5 interacting with the negatively charged moieties of LPS. The binding of LPS to HK was further shown to be maximal at pH 7.0 and in an isotonic environment. The binding of LPS to HK was blocked by HKH19, endotoxin free C1-Inhibitor, heparin, and *N*-acetylglucosamine glycan but not by lipid A with IC_{50}s of 15 nM, 20 μg/ml, 10 nM and 10 mM,

respectively. This novel study highlights the role HK plays in infection. This study proposes that HKH19, heparin and C1-Inhibitor present therapeutic potential for the treatment of hypotensive bacteremia and sepsis.

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LIST OF ABBREVIATIONS

Plasma Kallikrein Kinin System	(KKS)
Prekallikrein	(PK)
High Molecular Weight Kininogen	(HK)
Prolylcarboxypeptidase	(PRCP)
Bradykinin	(BK)
High Molecular Weight Kininogen Alternate	(HKa)
Human Umbilical Vein Endothelial Cells	(HUVEC)
Angiotensin II	(Ang II)
Angiotensin 1-7	(Ang 1-7)
Angiotensin III	(Ang III)
Angiotensin 2-7	(Ang 2-7)
Bradykinin B2 Receptor	(BKB2R)
Bradykinin B1 Receptor	(BKB1R)
Nitric Oxide	(NO)
Renin Angiotensin System	(RAS)
Lipopolysaccharide	(LPS)
Biotin-LPS	(BLPS)
Biotin-HK	(BHK)
19 Amino Acids of the HK Binding Site	(HKH19)

I. INTRODUCTION

A. Plasma Kallikrein System

The Plasma Kallikrein Kinin System (KKS) plays an important role in the activation and increase of the inflammatory response to tissue injury and in patients with septic shock, hypotensive bacteremia, in rhesus monkeys infected with *Salmonella typhimurium* and upon exposure to negatively charged surfaces.[5,6,10] Composed of three serine protease zymogens Prekallikrein (PK), Factor XII (FXII) and Factor XI (FXI) and a nonenzymatic procofactor High Molecular Weight Kininogen (HK), the KKS is induced at the onset of an inflammatory response by the binding of a HK-PK complex and FXII to the surface of an endothelial cell or other biological activating surface. Upon adhering to the cell membrane, FXII is converted to an alternate form (FXIIa) and PK is converted to Kallikrein by the serine protease Prolylcarboxypeptidase (PRCP) and FXIIa. The formed Kallikrein will then autodigest HK to release Bradykinin (BK) and an alternate form of HK (HKa).[3,5] HK is a multifunctional, multidomain β-globulin protein with a molecular weight of 120 kDa and has a physiological concentration of 80 μg/ml (670nM).[3] The activated FXIIa then aids in the conversion of Factor XI to Factor XIa. This activation induces the intrinsic coagulation pathway also known as the contact system.[5]

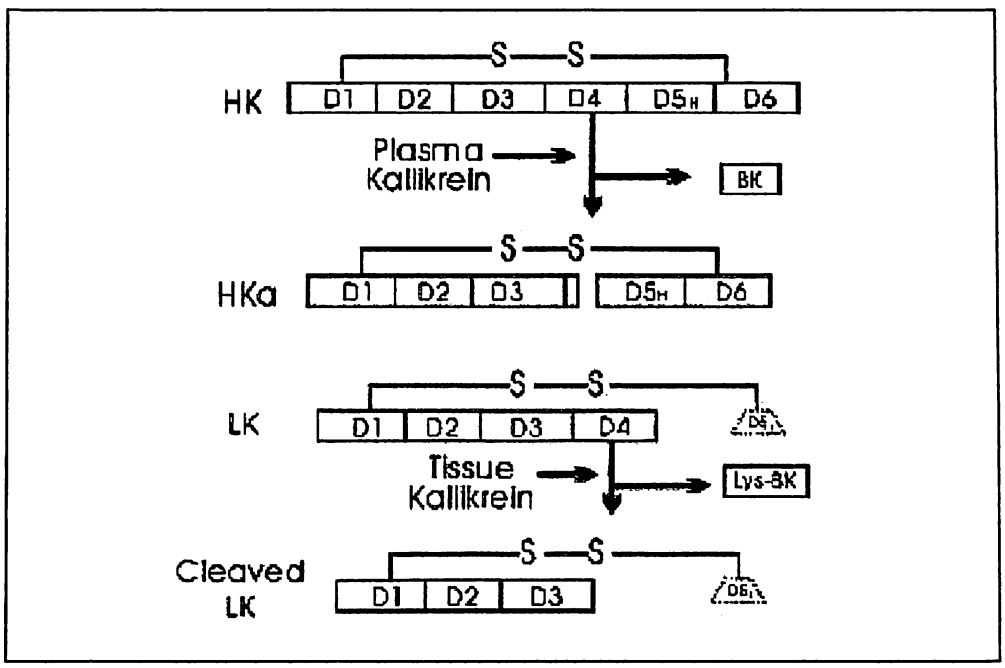

Figure 1: Autodigestion of High Molecular Weight Kininogen.

HK consists of 6 domains arranged into a single chain. HK is further divided into a heavy chain which consists of Domains 1 through Domain 3 and a light chain that contains Domain 5H and Domain 6. Domain 4 contains the amino acid sequence for BK which links the heavy chain and the light chain together. When PK is converted to Kallikrein by PRCP, the formed Kallikrein will cleave HK to break the disulfide bond between the heavy chain and the light chain to release BK.[3]

B. Prolylcarboxypeptidase

Prolylcarboxypeptidase (PRCP) is a serine protease commonly found in endothelial cells and plays an important role in modulating numerous vasoactive hormones and protein precursors that are involved in blood pressure regulation. Originally, PRCP was characterized as an exopeptidase that cleaves small, biologically active peptides at the carboxy terminus, typically the Pro-Phe bond, at neutral pH and at pH's of less than 7.0. When the serine protease was extracted and purified from human umbilical vein endothelial cells (HUVEC), it was found to be a PK activator. Prior experiments have shown that PRCP isolated from endothelial cell lysates, Chinese Hamster Ovary cell lysates and recombinant PRCP purified from Schneider 2 insect cells will only activate PK when coupled to its receptor on HK. *In vivo* control of PRCP utilizes the endogenous inhibitors: C1 inhibitor, $\acute{\alpha}_2$-macroglobulin and antithrombin III.[11] In addition, to catalyzing the conversion of PK to Kallikrein, PRCP has also been shown to catalyze the hydrolysis of angiotensin II (Ang II) to angiotensin 1-7 (Ang 1-7) and angiotensin III (Ang III) to angiotensin 2-7 (Ang 2-7).[7] This degradation of Ang II to Ang 1-7 has lead to PRCP's characterization as an Ang II degrading enzyme. Upon investigations of PRCP's catalytic ability on Ang II, PRCP was found to have a K_m of 200 μM.[13] Recent studies examining the colocalization of PRCP revealed that the protein is present on the cell surface and in lysosomes. The studies also revealed that PRCP colocalizes with gC1qR, urokinase plasminogen activator receptor (uPAR), and cytokeratin 1; however, does not completely colocalize with lysosomal associated protein.[8]

C. Bradykinin

Bradykinin plays an important role in the causation of vascular inflammation and in the regulation of local blood flow and blood pressure. BK is a vasodilator and a potent stimulator of tissue plasminogen activator release.[2] Under normal conditions in the cardiovascular and renal systems, blood pressure regulation occurs via the Bradykinin B2 receptor (BKB2R). BKB2Rs are found in abundance in the tissues of the cardiac, renal and vascular systems. Literature reports that BKB2Rs exert their effects by activating vasoactive components of the prostaglandin-Nitric Oxide cascade.[1]

Prior studies have shown that Bradykinin effects are mediated by the constitutively active BKB2R and by inducible Bradykinin B1 receptors (BKB1R). It has been shown that BKB1Rs are activated in cardiac, renal, and vascular tissues by stimuli including tissue damage, bacterial lipopolysaccharides, and cytokines. The BKB1Rs are most commonly induced during the inflammatory response and nociceptive reactions.[1,4] Like the BKB2Rs, the BKB1Rs are known to play an important role in normal blood pressure regulation. In pharmacological experiments utilizing genetically engineered BKB2R knockout mice, the mildly hypertensive rats have shown that increasing salt or exogenous angiotensin II concentrations induced greater levels of hypertension and also displayed higher levels of BKB1R gene activity.[4]

As mentioned earlier, the KKS plays an important role in responding to inflammation. The KKS can utilize two distinct pathways to respond to chronic inflammation and acute inflammation. In order to initiate an acute inflammatory response, the KKS system is activated. Leukocytes then marginate within the blood vessel and migrate towards the site of the inflammatory response. The stimulation of

BKB1R then causes cell adhesion molecules to become activated and play an important role in the regulation of the neutrophils' adhesion cascade. HKa then complexes to neutrophils and monocytes that are present at the site of the inflammatory response and inhibit their binding to fibrinogen. This prevents them from migrating and ensures their presence at the site of injury. Due to the chemotactic properties of Kallikrein, neutrophil aggregation is induced. This in turn stimulates the release of elastase and superoxide which serve as potent inducers of tissue injury. In addition, intracellular signaling and the release and synthesis of other mediators generating pain (prostaglandin release), vascular dilation (prostaglandin I_2), or permeability (endothelial nitric oxide synthase) occur due to activation of BKB2Rs and cell adhesion molecules.[3]

As a result of a chronic inflammatory response, HKa binds directly to monocytes and neutrophils on urokinase-type plasminogen activator receptors (uPAR) and Mac-I located on the surface of monocytes and neutrophils. This in turn upregulates the production and release of cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, chemokines monocyte chemoattractant protein (MCP)1 and IL-8 from monocytes and superoxide and elastase from neutrophils. These released factors play an essential role in inducing tissue injury and eventually chronic inflammation. Eventually, HKa is inactivated by proteases in the course of its biological nine hour half-life.[3]

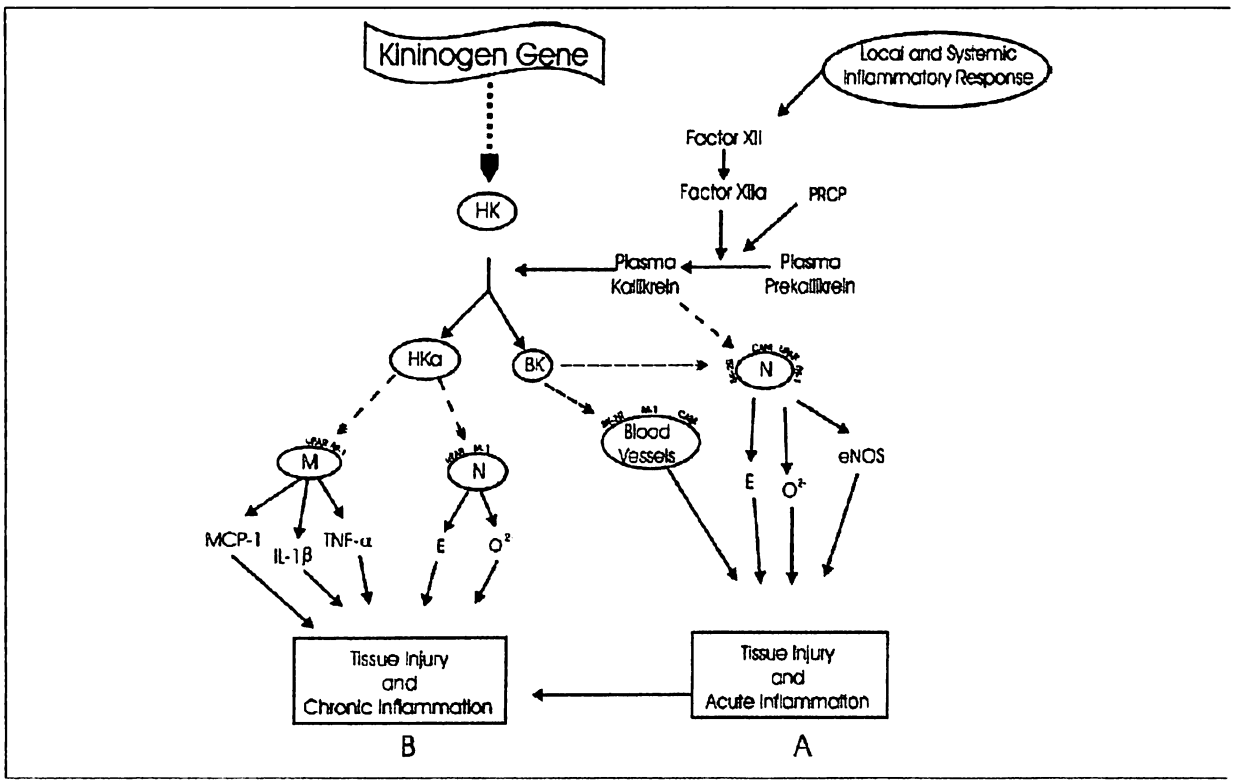

Figure 2: Role of the Kallikrein-Kinin System in inflammation.

The KKS can utilize two distinct pathways to respond to chronic inflammation and acute inflammation.

D. Nitric Oxide

Nitric Oxide (NO) is a two-atom compound that is synthesized physiologically by various tissue and cell types in the body and plays a complex role in physiology. In the cardiovascular system, NO acts as a mediator of vasodilation in the endothelium helping to regulate vessel tone, but also plays a role in the regulation of platelet aggregation, leukocyte adhesion, paracrine and autocrine pathways involving control of vascular cell growth and remodeling, endothelial cell apoptosis, migration and organization. NO synthesis from L-arginine in endothelial cells is catalyzed by the enzyme Nitric Oxide Synthase (eNOS). Studies have shown that physiologically eNOS is activated by increasing the levels of intracellular calcium present. The increasing calcium levels will in turn upregulate the binding of calmodulin to eNOS. eNOS, a transmembrane protein, is found on Golgi and caveolar membranes; however, the targeting of eNOS to caveolae is dependent upon co- and post-translational modification (myristoylation, palmitoylation) which are required for optimal enzymatic activity.[9]

E. Renin-Angiotensin System

The Renin-Angiotensin System (RAS), similar to Bradykinin, plays an important role in the regulation of blood pressure and the vascular response to injury. This relationship can be illustrated due to the dual roles of RAS in the homeostasis of salt and water in the body's response to vascular injury. Damage to the endothelial wall can be caused due to the increased mechanical strain that is associated with high blood pressure. When RAS is activated, the blood pressure is increased which leads to the causation of a local inflammatory response to aid in repairing the injury. Due to elevated blood

pressure and angiotensin II-induced oxidative stress, continual response to this injury leads to microvascular damage and endothelial dysfunction. This response has pathophysiological effects increasing the chances of myocardial infarction, heart failure, stroke, peripheral arterial disease, and renal disease.[11]

The Renin Angiotensin System plays an important role in protecting the heart, endothelium, brain and kidney from sustained exposure to blood pressures that exceeds the physiological range through a complex system of neuroendocrine interactions. Continual activation of RAS results in the causation of hypertension and activates a cascade of proinflammatory, prothrombotic and atherogenic effects. The synthesis of the central metabolite Ang I is largely dependent upon the activity of renin. Renin, an enzymatic hormone that plays an important role in salt regulation by the kidneys, is synthesized and released by the liver to counter act decreases in renal perfusion pressure. This released renin then catalyzes the conversion of angiotensinogen into the inactive peptide Angiotensin I (Ang I, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). As the central metabolite of RAS, Ang I can then follow three different paths. Ang I can be converted into the vasoactive peptide Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) by angiotensin-converting enzyme (ACE). In this conversion, ACE cleaves the His-Leu residues from the carboxy terminus of the peptide. Surprisingly, not all of the conversion from Ang I to Ang II is done by ACE. In fact, nearly ~ 40% of the conversion is catalyzed through chymase, cathepsin G, and other serine proteases. The formed Ang II is then known to interact with at least 2 different receptors, angiotensin II receptor subtype 1 (AT_1) and angiotensin II receptor subtype 2 (AT_2). Of the two Ang II receptor subtypes, the AT_1 receptor has been more readily characterized and is understood in

greater depth. Widely distributed throughout the body, the AT_1 receptors with ligand bound leads to vasoconstriction, release of aldosterone by the adrenal glands, promotes the retention of salt and water and activates vascular remodeling. Since activation of the AT_1 receptors causes increases in oxidative stress and transforming growth factor β, it contributes to the proinflammatory, atherogenic, and prothrombotic environment. The binding of Ang II to AT_1 receptors can be blocked by the AT_1 antagonist, angiotensin receptor blocker.[12]

Although not as readily understood, the AT_2 receptor is normally activated as a result of tissue injury and exerts its effects on cardiovascular tissue that are counterregulatory to those regulated by AT_1 receptors.[12] The exact effects of the AT_2 receptor have not been completely characterized; however, experiments involving AT_2-deficient mice revealed that these receptors are involved in regulating the cellularity of atherosclerotic lesions. In studies where the AT_2 receptor was overexpressed, an attenuation of perivascular and cardiac fibrosis was observed upon exposure to Ang II. In addition, the activation of the AT_2 receptor may also be beneficial due to the activation of the KKS.

In addition to Ang I being converted to Ang II, Ang I can also be converted to Ang 1-7 by neutral endopeptidase. The formed Ang 1-7 then binds to the $AT_{1\text{-}7}$ receptor which plays an important role in activating angiotensin-converting enzyme 2 (ACE2). The activation of ACE2 is counterbalanced by the AT_1 receptor which plays an important role in inhibiting the activity of ACE2. ACE2 plays an important role in the regulation of Ang II to Ang 1-7. If ACE2 is activated by the $AT_{1\text{-}7}R$, then it can catalyze the conversion of Ang II to Ang 1-7. However, if the AT_1 receptor is activated, ACE2 is

inhibited, thus preventing the conversion of Ang II to Ang 1-7. The third fate of Ang I is its conversion to Ang 1-9 by ACE2.[12]

F. Kallikrein-Kinin System Counterbalances Renin-Angiotensin System

As mentioned earlier, the KKS plays an important role in RAS by acting as an essential counterbalance, due to the ability of PRCP to interact in the KKS or RAS, the role of ACE, the interactions of Bradykinin and Ang 1-7 and the differing effects of activating AT_1 and AT_2 receptors. As stated earlier, PRCP is a known PK activator converting PK to Kallikrein, which causes HK to be cleaved releasing BK and HKa. However, PRCP is also an important player in activating RAS. When PRCP converts PK into Kallikrein, it also catalyzes the conversion of Prorenin to Renin initiating the RAS cascade. In addition, PRCP plays an important role further down the cascade due to its ability to convert Ang II to Ang 1-7. Although PRCP plays a role in this conversion, ACE2 is 100-fold kinetically better at degrading Ang II to Ang 1-7 with a K_m of inactivation of 2 mM. This relationship is significant due to the end physiological result of each system. By activating KKS, vasodilation occurs due to the production of NO and prostacylin, where as activating RAS can lead to vasodilation or vasoconstriction. If RAS is activated and PRCP is inhibited, Ang II will lead to vasoconstriction through the AT_1 Receptor. However, if RAS and PRCP are activated, Ang II will be converted to Ang 1-7, which will lead to vasodilation and formation of NO and prostacyclin. This relationship allows for the KKS to counterbalance RAS.[13]

In addition, the KKS serves as a counterbalance to RAS due to the interaction between Ang 1-7 and BK. The best studied interactions between Ang 1-7 and the KKS

G. Lipopolysaccharide

Lipopolysaccharide (LPS) is an endotoxin that encompasses areas of the cell wall in Gram-negative bacteria. When LPS is administered to an organism, it has the potential to induce an inflammatory response, which if severe enough can lead to multiple organ disfunctions, septic shock and death. In addition to its location in the cell wall of Gram-negative bacteria, LPS is an immune stimulant which induces the release of (TNF)-ά, IL-1 and IL-6. These pro-inflammatory cytokines play an essential role in mediating the physiological effects of LPS and are involved in the modulation of the central nervous system-mediated responses.[14]

H. Experimental Rationale

Although the KKS can perform differently in response to different conditions, PK can only be activated when complexed to HK on the cell membrane surface or when bound to a negatively charged moiety. Since LPS contains a negative surface and is capable of eliciting an inflammatory response, studies were conducted to characterize the interaction between LPS and HK. The findings of this study may play an integral role in the development of new drug therapies to treat infection.

II. MATERIALS AND METHODS

Materials.

Prekallikrein (PK) with a specific activity of 27 U/mg was purchased from Enzyme Research Laboratories (South Bend, IN). S2302, a chromogenic substrate, was purchased from Chromogenics, Inc. (Lexington, Mass). Single chain High Molecular Weight Kininogen (HK) with a molecular weight of 120 kDa on reduced SDS-PAGE and a specific activity of 13 U/mg in acetate buffer (4 mM sodium acetate-HCl and 0.15 M NaCl, pH 5.3) was purchased from DiaPharma Laboratories, Inc. (West Chester, OH).

Binding of biotin-HK to LPS.

The biotinylated binding studies were conducted by adding LPS at 2 μg/ well in a microtiter plate and incubating overnight at 4° C with 0.1 M Na_2CO_3, pH 9.6. The next day, the wells were blocked with 1% gelatin for 1 hour at 37° C. After 1 hour, the cells were washed with Hepes-$NaHCO_3$ buffer [137 mM NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 14.7 mM Hepes, 5.5 mM Dextrose, and 0.1% gelatin, pH 7.1] containing 2 mM $CaCl_2$, and 1 mM $MgCl_2$, blocked with 1% gelatin at pH 7.1 in the presence and absence of 50-fold molar excess HK and then incubated with 20 nM biotin-HK for 1 hour at 37° C. The binding of biotin-HK to LPS was ascertained using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxidase-specific fast reacting substrate, 3,3',5,5'-

tetramethylbenzidine dihydrochloride (TMB) as suggested by Pierce (Rockford, IL). The binding of biotin-HK to LPS was determined quantitatively by measuring the absorbance of the reaction mixture in a microplate auto reader ELX 800 Bio-Tek Instrument (Winooski, VT) at 450 nm. The total binding of biotin-HK to LPS was determined and compared with the level of binding observed in the presence of a 50-fold molar excess of HK.

Binding of biotin-LPS to HK.

A 96 well microtiter plate was coated with 2 μg of HK in 0.1 M Na_2CO_3, pH 9.6 overnight at 4° C. The next day, the wells were washed three times with Hepes-$NaHCO_3$ buffer containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ and then blocked with 1% gelatin for 1 hour at 37° C. After blocking, the HK bound to the microtiter plate was washed and then incubated in the absence and presence of increasing concentrations of biotin-LPS in Hepes-$NaHCO_3$ buffer for 1 hour at 37°C. The binding of biotin-LPS to HK was ascertained using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxidase-specific fast reacting substrate, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB). The binding of biotin-LPS to HK was determined quantitatively by measuring the absorbance of the reaction mixture in a microplate auto reader at 450 nm. The total binding of biotin-LPS to HK was determined and compared with the level of binding observed in a 100-fold molar excess of LPS.

Inhibtion of biotin-LPS binding to HK.

2 μg of HK in 0.1 M Na_2CO_3, pH 9.6 was coupled to a 96 well microtiter plate and incubated overnight at 4°C. The next day the wells were blocked with 1% gelatin for 1 hour at 37° C. After blocking, the microtiter plate wells were washed and incubated with 3-5 μg/ml of biotin-LPS in Hepes-carbonated buffer in the absence or presence of various concentrations of HKH19 [19 amino acid residues of HK's domain 5, diethyl pyrocarbonated HKH19 (modified HKH19), endotoxin free heparin, C1-inhibitor, cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR), glucose, fucose, sucrose, *N*-acetylglucosamine gylcan, and lipid A for 1 hour at 37° C. After incubation, the binding of biotin-LPS to HK was ascertained using ImmunoPure streptavidin horseradish peroxidase conjugate and peroxidase-specific fast reacting substrate, 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB). The binding of biotin-LPS to HK was determined quantitatively by measuring the absorbance of the reaction mixture in a microplate auto reader at 450 nm.

Endothelial cell culture.

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA) and cultured according to the vendor's instructions. Confluent cells (>3.0 x 10^4/cm^2) at passage numbers one to five were used in the assays. Lipopolysaccharide (*Escherichia coli* serotype 0111:B4) was diluted in endothelial cell growth medium and exposed to cells between 1 and 24 hours with a final concentration of 1-1000 ng/ml.

Statistical analyses.

Results are expressed as means ± SEM, and data was analyzed using Student's *t*-test for significant difference. Statistical significance was defined as $P \leq 0.05$.

III. RESULTS

A. Characterization of LPS interaction with HK

i. Time-course of biotin-LPS binding to HK: Since PK can be activated only by PRCP when properly assembled to HK on the cell membrane surface or on a negatively charged moiety, the effect of LPS on the assembly of the HK-PK complex and the activation of the complex on endothelium was characterized. Studies were initially conducted to characterize the interactions that occur between biotinylated-LPS (BLPS) and HK. In these studies, 2 μg/well of HK or BSA was coupled to a 96 well microtiter plate overnight and was followed by the addition of BLPS. The addition of 5 μg/ml of BLPS to the microtiter plate containing HK resulted in increased binding between BLPS and HK over time (Figure 3). However, incubation of BLPS with the microtiter plate covered with BSA did not result in increased binding over time. These data suggest that LPS binds to HK and that the binding occurs in a time-dependent manner.

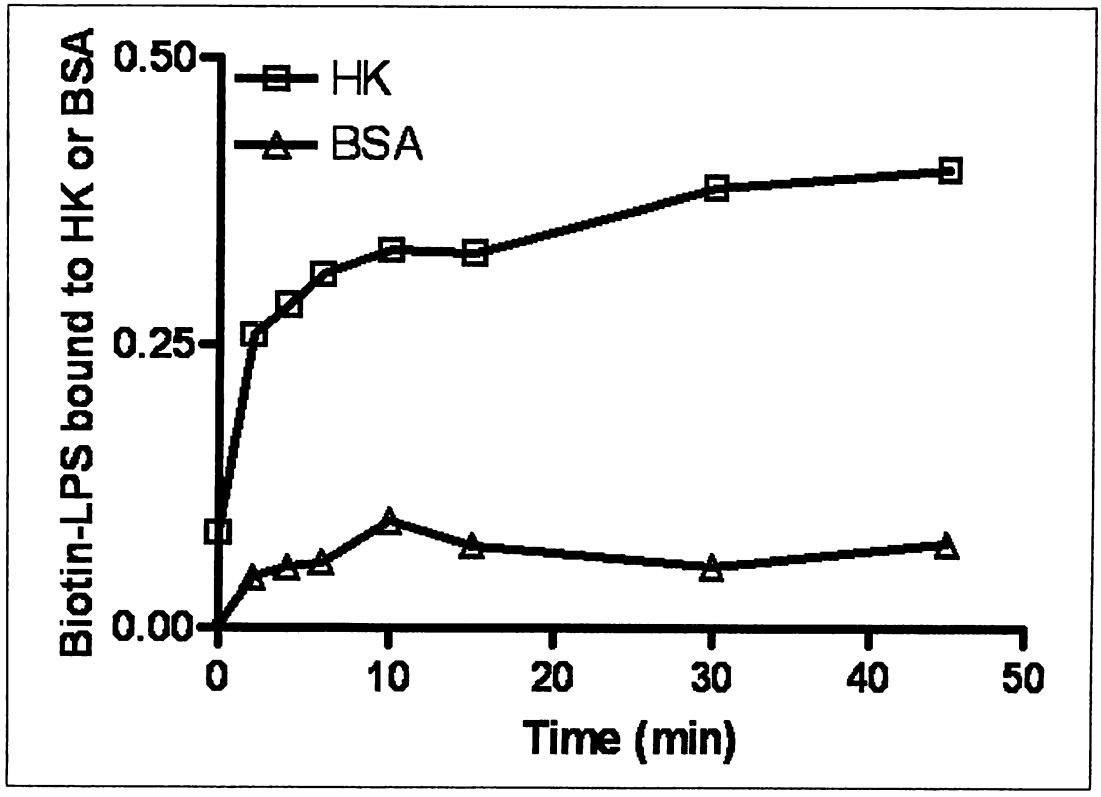

Figure 3: Time Course of biotin-LPS binding to HK.

Investigations were performed to determine if biotin-LPS could bind HK. HK or BSA was coupled to microtiter plates followed by treatment with biotin-LPS over a period of time. The amount of bound BLPS was then measured by the absorbance of the reaction mixture at 450 nm.

ii. Influence of pH and osmolarity on the binding of biotinylated-LPS to HK:

According to literature, suggestions have been made that the pH at the site of inflammation could be slightly acidic with a pH of about 6.5. In order to characterize the optimum pH for binding of LPS to HK, studies were conducted at various pH ranges and at different salt concentrations. When the pH of the buffer was adjusted, there was a marked change in the binding constant of LPS to HK (Figure 4). According to the pH studies, the binding of LPS (5 μg/ml) to HK (2 μg/ml) coated to the microtiter plate was maximal at pH 6.8-7.0.

When salt concentrations were increased from a hypotonic environment (200 mOsm) to an isotonic environment (335 mOsm) the binding of LPS (5 μg/ml) to HK (2 μg/ml) exhibited an increase in the binding affinity (Figure 5). The highest binding of LPS to HK occurred at 335 mOsm. When the salt concentrations were altered, LPS binding to HK was inhibited at high salt concentrations. The data from this study also showed that the binding of LPS to HK in a 96 well microtiter plate was optimal at physiological salt concentrations. These data suggest that optimal binding of LPS to HK occurs in a slightly acidic and isotonic environment.

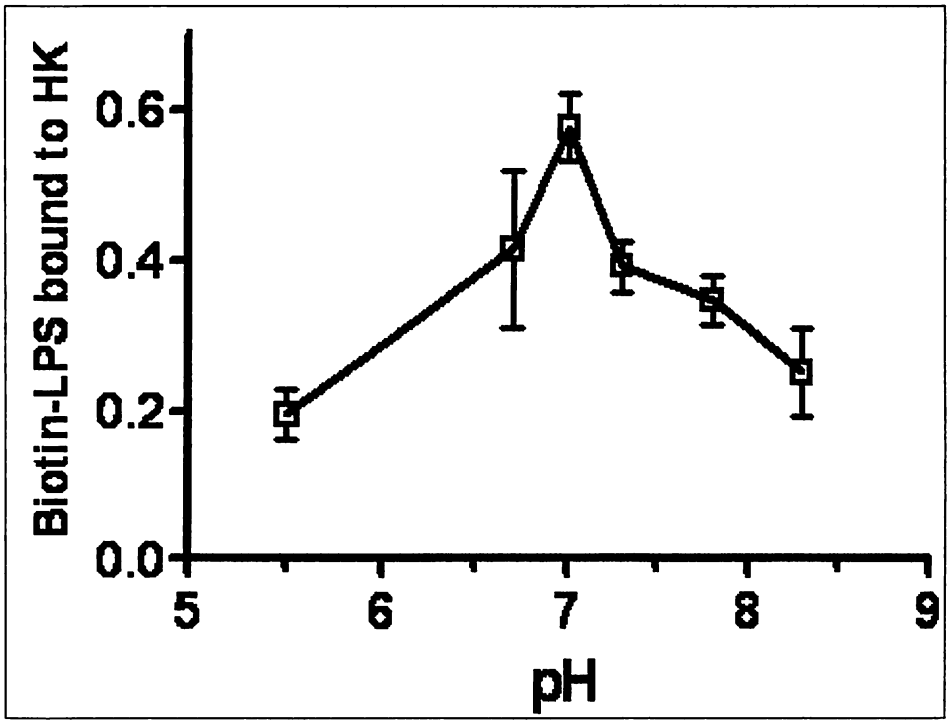

Figure 4: The influence of pH on biotin-LPS binding to HK.

In these studies the binding of biotin-LPS (5 μg/ml) to HK (2 μg/ml) that was linked to a 96 well microtiter plate was investigated under different pH conditions. The amount of bound BLPS was then measured by the absorbance of the reaction mixture at 450 nm.

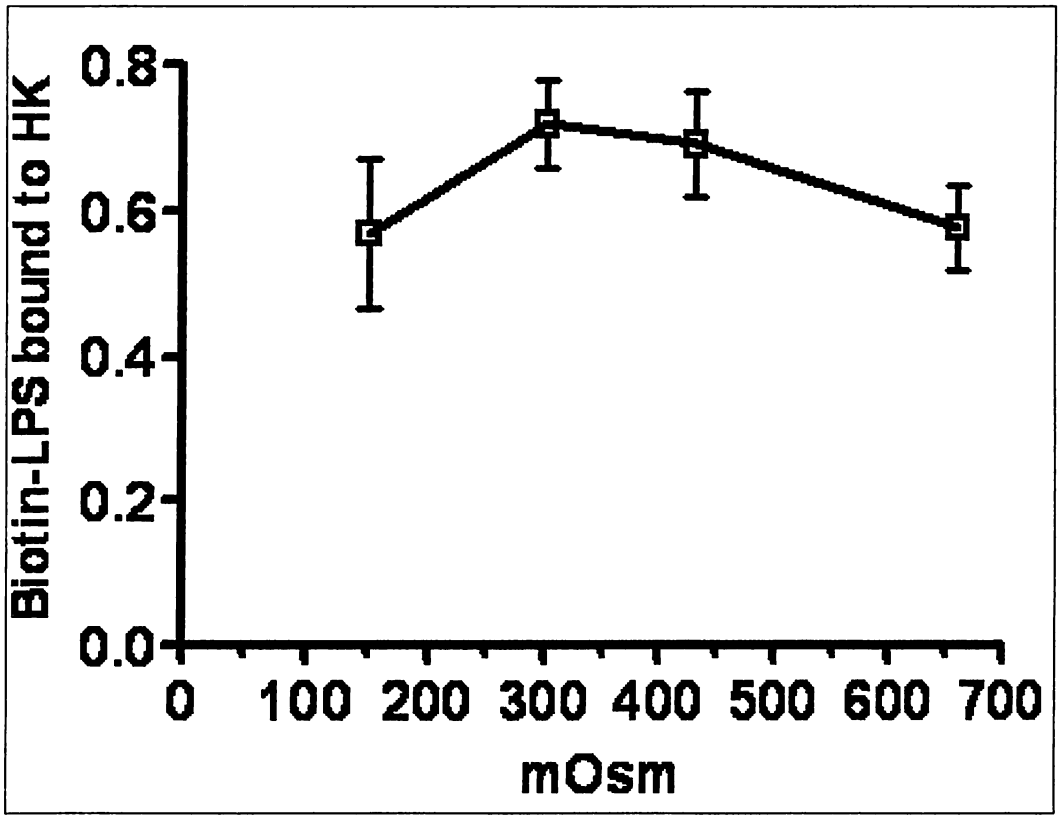

Figure 5: The effect of osmolarity on biotin-LPS binding to HK.

In these studies the effect of biotin-LPS (5 μg/ml) binding to HK (2 μg/ml) linked to a 96 well microtiter plate was investigated as varying osmolarities. The amount of bound BLPS was then measured by the absorbance of the reaction mixture at 450 nm.

iii. Determination of the binding of biotinylated LPS (BLPS) to HK: Since prior studies revealed that LPS binding to HK occurred in a time-dependent manner at an optimum pH and osmolarity, studies were performed to determine whether the binding of LPS to HK occurred in a dose-dependent manner. In doing this, HK or BSA was coupled to a 96 well microtiter plate and then treated with 0.0 to 10.0 μg/ml concentrations of BLPS. The data from this study showed that the binding of BLPS to HK was saturable and that half maximal binding occurred at 0.7 μg/ml (Figure 6). When a 96 well microtiter plate containing HK was treated with 0.0 to 10.0 μg/ml of BLPS and a fifty molar excess of HK, BLPS binding to HK linked to the plate was blocked. This interaction suggests that the binding was specific.

In order to determine if HK binding to LPS was dose-dependent 2 μg/ml of LPS was linked to a 96 well microtiter plate and incubated with 0.0 to 10 nM biotin-HK (Figure 7). Similar to the results of the previous study, the binding of biotin-HK to LPS bound to the microtiter plate was found to be saturable and that half maximal binding occurred at 6 nM (0.7 μg/ml). Likewise, when BSA was treated with increasing concentrations of biotin-HK little binding occurred between the BSA and HK. These combined data suggested that LPS would bind to HK. However, this finding may not reflect their actual physiological interaction due to the fact that LPS bound to HK at concentrations where LPS is 100-fold higher than it is found in the blood of patients with sepsis or in the plasma.

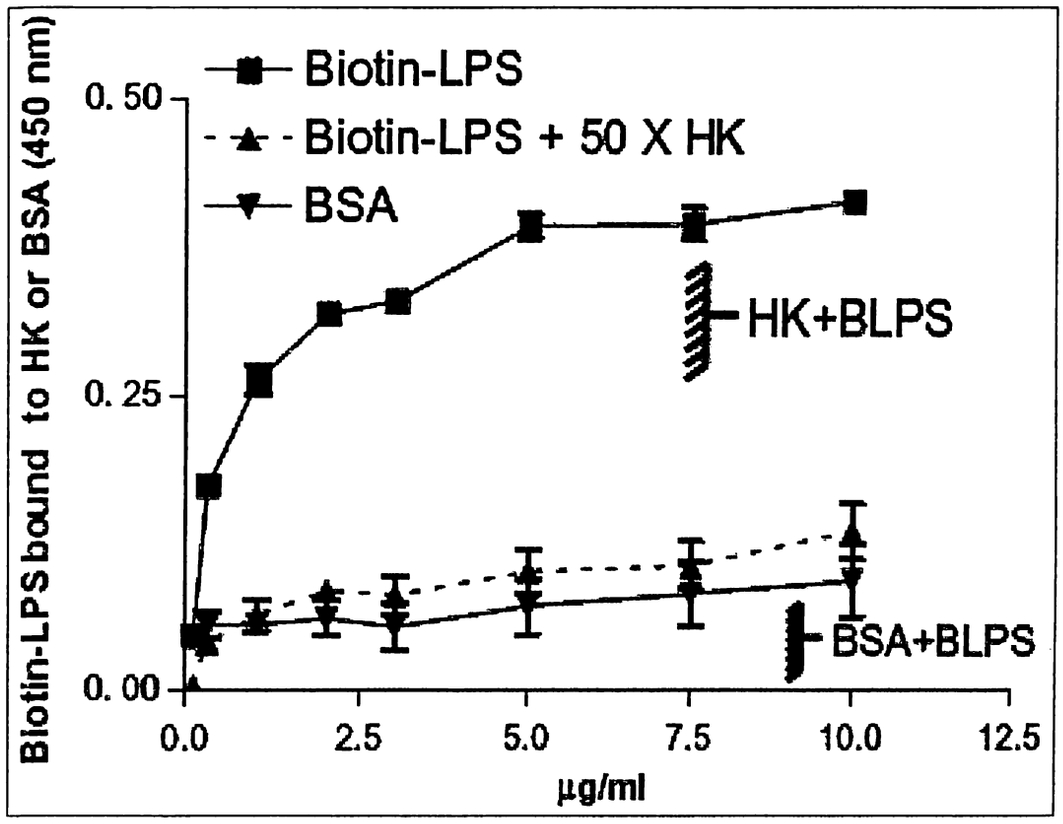

Figure 6: Binding Studies with Biotin-LPS.

In these studies 10 ng/ml to 10 μg/ml of biotin-LPS (BLPS) or BLPS with a 50 M excess of HK were incubated with 2 μg/well of HK or BSA linked to a 96 well microtiter plate at 37°C. The amount of bound BLPS was then measured by the absorbance of the reaction mixture at 450 nm.

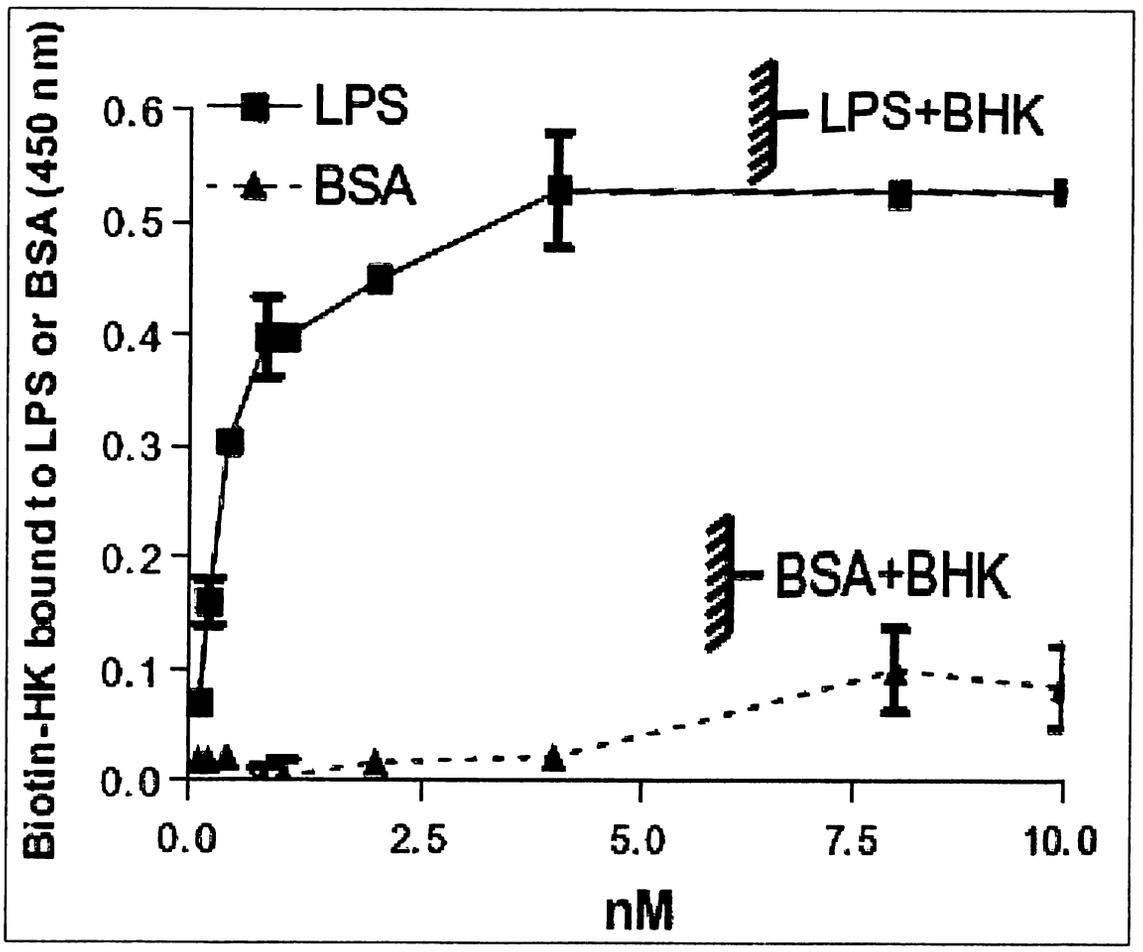

Figure 7: Binding Studies with Biotin-HK.

In these studies 0.01 to 10 nM of biotin-HK (BHK) were incubated with 2 μg/well of LPS or BSA linked to a 96 well microtiter plate at 37°C. The amount of BHK that was bound to LPS was then calculated by measuring the absorbance at 450 nm of the reaction mixture.

B. Inhibition Studies

i. Mapping LPS binding site(s) on HK: Previous studies have demonstrated that HK can bind to various unrelated proteins found on endothelial cells via the cell binding domain, HKH19. In order to determine if LPS binding to HK occurred through HKH19, HK (2 μg/ml) was linked to a 96 well microtiter plate and treated with LPS (3 μg/ml) and increasing concentrations of HKH19 (Figure 8). HKH19 was found to inhibit the binding of LPS to HK in a dose-dependent manner.

Due to the belief that hydrophobic amino acid residues are vital for LPS interaction with the target protein, studies were performed to assess whether the positive amino acid residues on HKH19 could non specifically attract the negatively charged moiety of LPS. In this study, histidine residues on the HKH19 were covalently modified to *N*-ethyloxyformyl-histidine by using diethyl pyrocarbonate. HKH19 was incubated with 1 mM diethyl pyrocarbonate at room temperature for 5 hours. The free diethyl pyrocarbonate was extracted by overnight dialysis in gelatin free Hepes-$NaHCO_3$ buffer. When increasing concentrations of modified HKH19 was added with LPS (3 μg/ml) to HK (2 μg/ml) linked to a 96 well microtiter plate, the modified HKH19 blocked the binding of LPS to the linked HK in a dose-dependent manner. HKH19 and modified HKH19 inhibited the binding of biotin-LPS to HK with IC_{50}s of 20 nM and 50 nM, respectively. The data from these studies suggested that LPS primarily binds to lysine amino acid residues that are present within the HKH19.

Since the negatively charged moiety of LPS was shown to non-specifically attract HKH19, the effect of heparin and C1-Inhibitor, positively charged species, were determined on LPS binding to HK. When heparin was added in increasing

concentrations it was shown to inhibit the binding of LPS to HK with an IC_{50} of 0.3 U heparin/ml (Figure 9). Furthermore, when heparin was administered in the presence of physiological concentration of HK (600 nM), it was shown to inhibit LPS's ability to bind to HK linked to the microtiter plate. In agreement with the literature, this finding suggested the high affinity of heparin for the domain 5 of HK. Since heparin possesses a high content of anionic sulfates and carboxylates, these data confirmed our hypothesis that the interaction of lysine residues of HKH19 to heparin or to LPS is an ionic interaction.

When C1-Inhibitor, which possesses a net cationic charge, was added in a dose-dependent manner it was shown to block LPS binding to HK linked to a 96 well microtiter plate and possessed an IC_{50} of 20 μg/ml (Figure 10). This result suggests that the binding between LPS and C1-Inhibitor is a direct interaction.

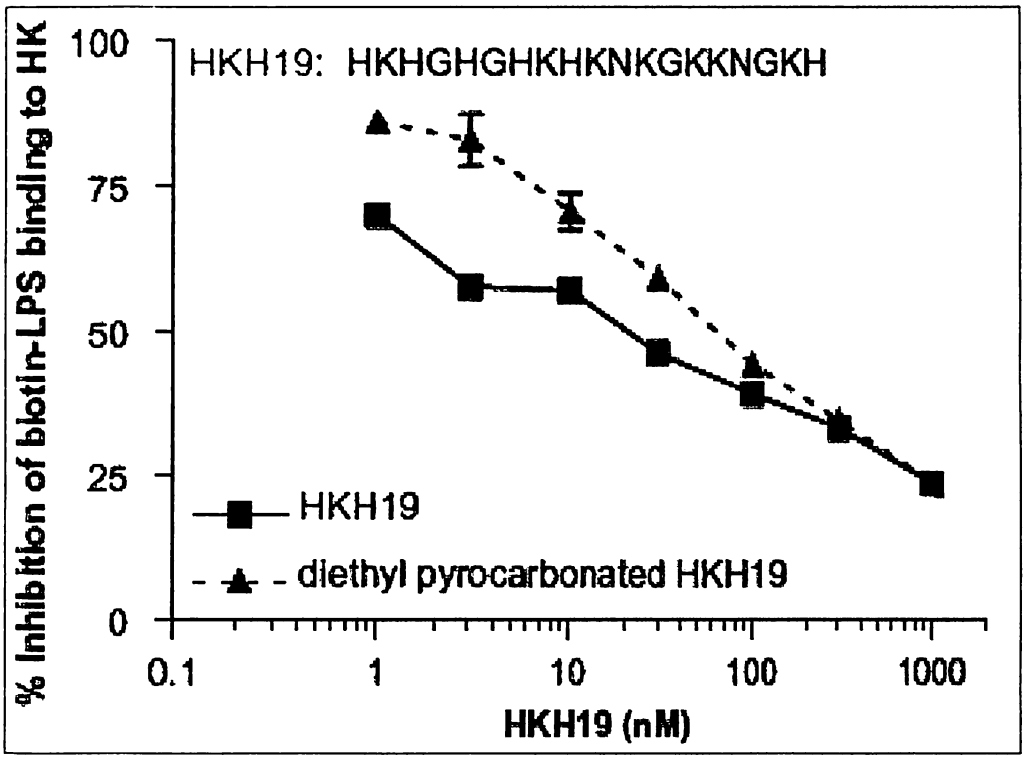

Figure 8: The effect of HKH19 on the binding of LPS to HK.

In this study HKH19 or diethyl pyrocarbonated HKH19 was reported to block LPS binding to HK. 0.001 to 1 μM of HKH19 or diethyl pyrocarbonated in the presence of biotin-LPS (BLPS) was incubated with 2 μg/well of HK that was linked to a 96 well microtiter plate. The amount of BLPS that was bound to HK was then calculated by measuring the absorbance of the reaction mixture at 450 nm.

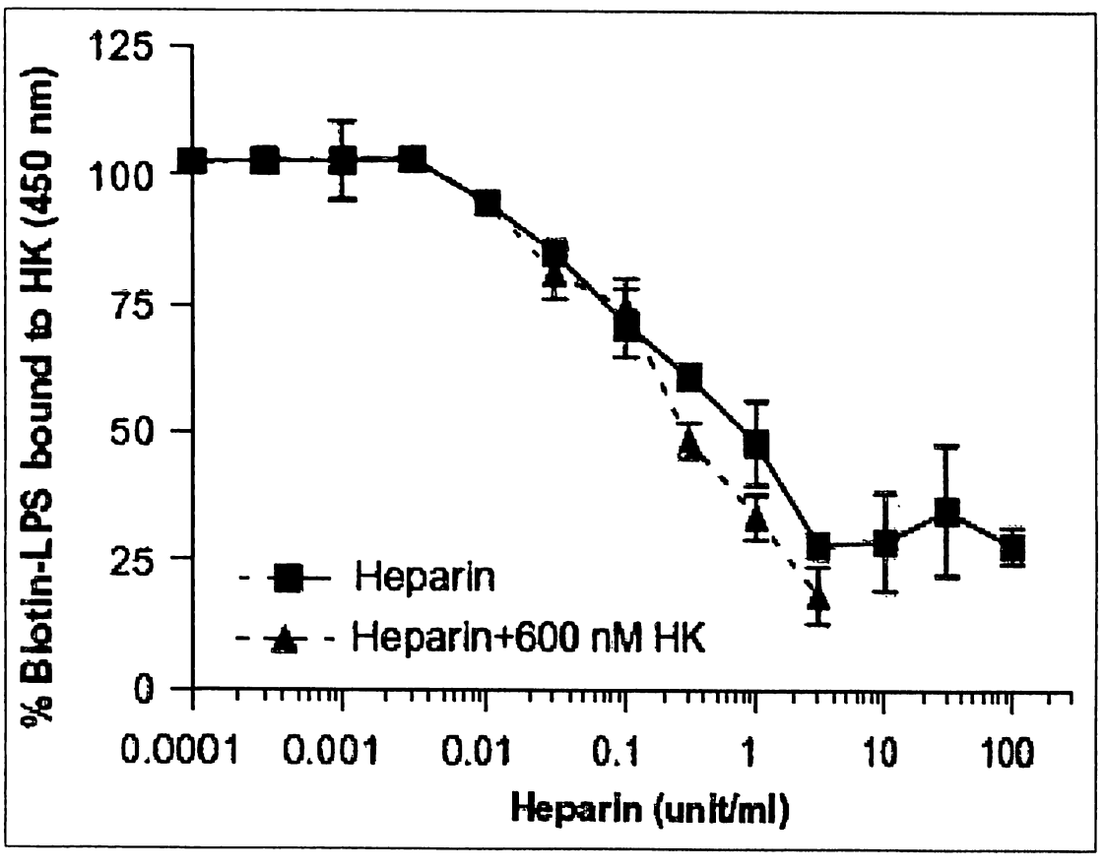

Figure 9: The effect of Heparin on LPS binding to HK.

In this study Heparin or Heparin with 600 nM HK was reported to block LPS binding to HK. 0.0001 to 100 unit/ml of endotoxin free heparin or heparin plus 600 nM HK in the presence of BLPS was incubated with 2 μg/well of HK that was linked to a 96 well microtiter plate. The amount of BLPS that was bound to HK was then calculated by measuring the absorbance of the reaction mixture at 450 nm.

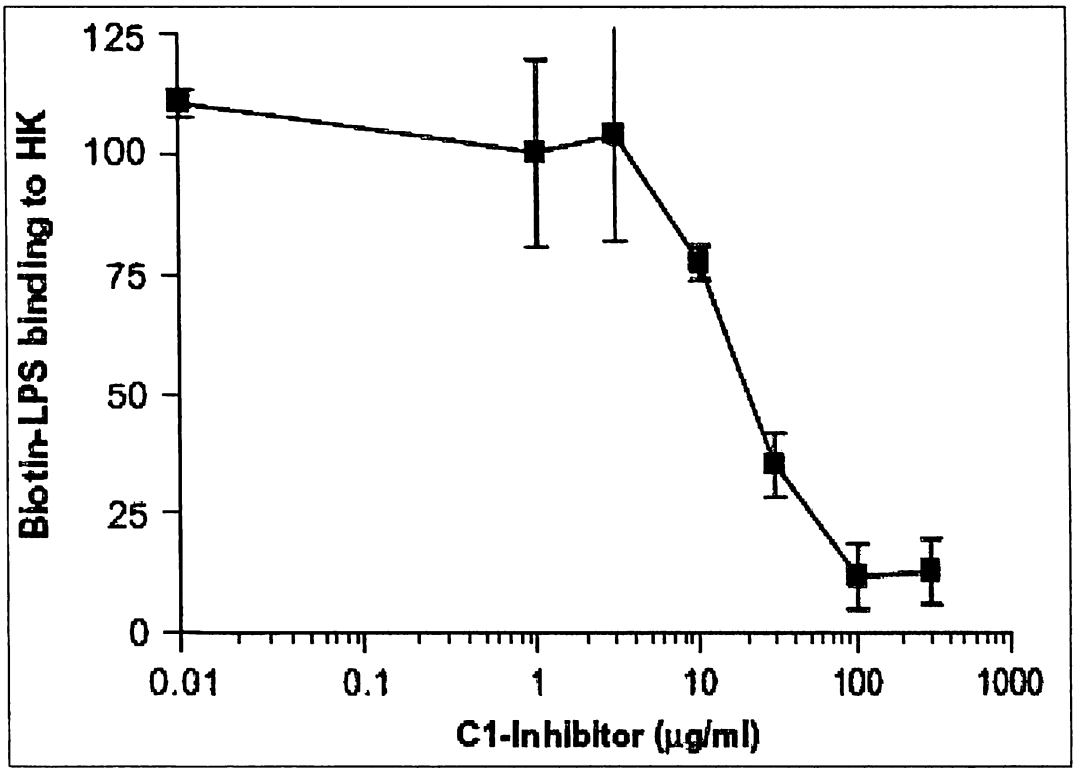

Figure 10: The effect of C1-Inhibitor on LPS binding to HK.

In this study C1-Inhibitor was reported to block BLPS binding to HK. Various concentrations of C1-Inhibitor in the presence of BLPS was incubated with 2 µg/well of HK that was linked to a 96 well microtiter plate. The amount of BLPS that was bound to HK was then calculated by measuring the absorbance of the reaction mixture at 450 nm.

ii. Mapping HK binding site(s) on LPS: Next studies were conducted to determine the HK binding site(s) on LPS. Since LPS is composed of a carbohydrate moieties, the effects of monosaccharide, disaccharide, and polysaccharide were investigated to determine its effect of LPS binding to HK. Studies investigating the effects of glucose, fucose, sucrose, deoxyglucose, and galactose showed little effect on the binding of LPS (3 μg/ml) to HK (2 μg/ml) that was linked to the microtiter plate. When *N*-acetylglucosamine glycan was investigated, it blocked the binding of biotin-LPS to HK in a dose-dependent manner (Figure 11). Furthermore, *N*-acetylglucosamine glycan inhibition possessed an IC_{50} of 10 mM.

Next studies these studies were repeated to determine if these carbohydrates and lipids would effect HK binding to LPS linked to a microtiter plate. Likewise, glucose, fucose, sucrose, deoxyglucose and galactose again had little effect of HK (2 μg/ml) binding to LPS (3 μg/ml) that was linked to microtiter plate (Figure 12). Again, when *N*-acetylglucosamine glycan was investigated, it blocked the binding of biotin-HK to LPS linked to microtiter plate in a dose-dependent manner and possessed an IC_{50} 200 mM.

Due to the fact that LPS also contains Lipid A moieties, studies were conducted to determine the effect of different concentrations of Lipid A on the binding of biotin-LPS to HK linked to microtiter plate. Data from this study reveal that the binding of biotin-LPS to HK was unchanged by the presence of Lipid A (Figure 13). The data from these studies implied that the main site in which HK binds to LPS is the polysaccharide side-chains of LPS.

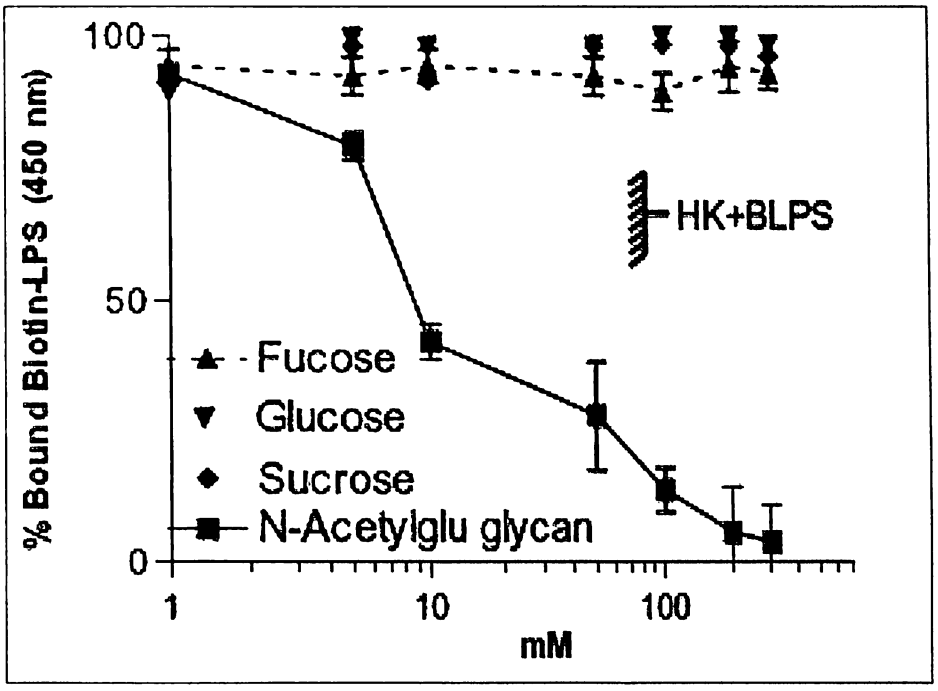

Figure 11: The effect of sugars on LPS binding to HK.

In this study increasing concentrations of fucose, glucose, sucrose and acetyl glucosamin glycan in the presence of BLPS was incubated with 2 μg/well of HK that was linked to a 96 well microtiter plate. The amount of BLPS that was bound to HK was then calculated by measuring the absorbance of the reaction mixture at 450 nm.

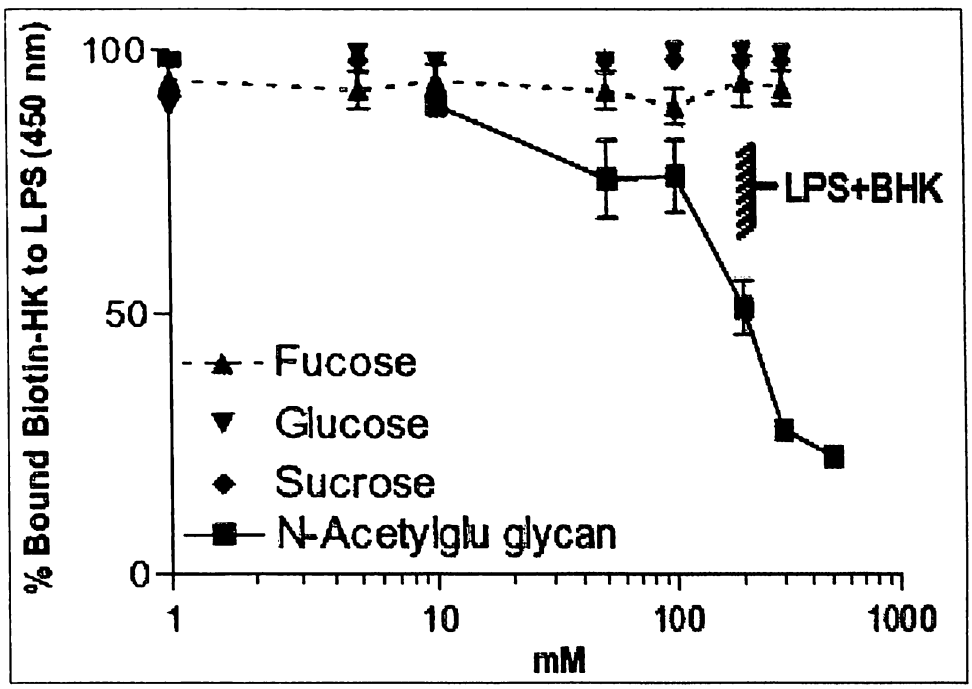

Figure 12: The effect of sugars on HK binding to LPS.

In this study increasing concentrations of fucose, glucose, sucrose and acetyl glucosamin glycan in the presence of BHK was incubated with 2 μg/well of LPS that was linked to a 96 well microtiter plate. The amount of BHK that was bound to LPS was then calculated by measuring the absorbance of the reaction mixture at 450 nm.

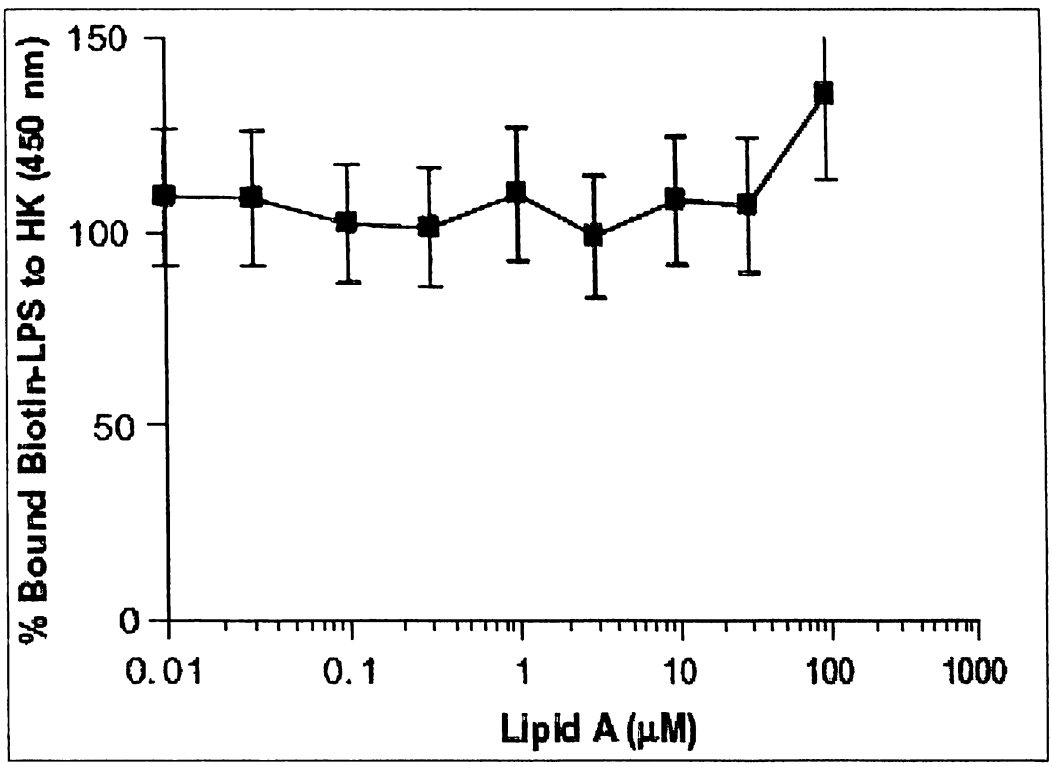

Figure 13: The effect of Lipid A on LPS binding to HK.

In this study increasing concentrations of Lipid A in the presence of 20 nM BLPS was incubated with 2 μg/well of HK that was linked to a 96 well microtiter plate. The amount of BLPS that was bound to HK was then calculated by measuring the absorbance of the reaction mixture at 450 nm.

IV. DISCUSSION

Since PK can only be converted to Kallikrein when HK is bound to the cell membrane surface or an artificial negative surface and LPS possesses a negatively charged domain with the ability to induce an inflammatory response in humans, the interaction of LPS and HK was determined. In this study we show that the binding of LPS to HK occurs in a time dependent, dose dependent, saturable and reversible manner.

The interaction of LPS on HK was shown to occur via the positively charged Lysine residues on domain 5. Furthermore, this interaction can be inhibited by HKH19, diethyl pyrocarbonated HKH19, and heparin. In addition, this study shows that the HK binding site on LPS is the polysaccharide chain. Inhibition of this interaction occurred by *N*-acetylglucosamine glycan and C1-Inhibitor. These data reveal that LPS binds to HK and this interaction possesses insights that might potentially be utilized in treating patients with hypotensive bactermia and sepsis.

In order to further characterize this interaction, a set of competitive inhibition studies were undertaken based on the prior data that was collected. Our data suggests that the attraction of C1-Inhibitor for LPS outcompetes the binding of LPS to HK. It is reasonable to speculate that this interaction of C1-Inhibitor with LPS might lead to the continual amassing of Kallikrein and activated FXII during endotoximea. In addition, the ability of heparin to inhibit LPS binding to HK was not affected in the presence or absence of physiological concentration of HK (600 nM). This data could be applicable to

future studies to determine if HK is involved in the removal of free heparin from systemic circulation in patients suffering from sepsis.

The activation of the Kallikrein Kinin System plays an important role in hypertension and inflammation. Therefore, the KKS metabolites play an integral role in health and disease. Since the KKS system is responsible for the production of BK and HKa, our data emphasizes the importance of HK, BK, and HKa in infection. However, further investigations need to be undertaken in order to characterize its predictive values in inflammation.

Furthermore, our data further provides evidence that HK plays a protective role in the coagulation system against infection. Our interesting and novel method of characterizing the inhibition of microbial binding (via HKH19, heparin, and C1-inhibitor) to the cell surface, should be further investigated with clinical studies, as it possesses potential therapeutic implications for patients with sepsis and hypotensive bactermia.

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