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GHRELIN DECREASES MICROVASCULAR LEAK DURING INFLAMMATION

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Abstract

Background—Obesity is a risk factor for poor outcomes after trauma and circulating levels of ghrelin are decreased in obese patients. We hypothesized that ghrelin modifies microvascular permeability. The purposes of this study were to determine: 1) the effect of ghrelin on microvascular permeability, 2) the effect of ghrelin on microvascular permeability during lipopolysaccharide (LPS)-induced inflammation, 3) the involvement of the GHS-R1a cell receptor, and 4) the involvement of NF-κB.

Methods—Hydraulic permeability (L_p), a measure of trans-endothelial fluid leak, was measured in rat mesenteric post-capillary venules. L_p was measured during continuous administration of 1) ghrelin (3 μM), 2) ghrelin and systemic LPS (10mg/kg), 3) the GHS-R1a receptor antagonist, [D-Arg1 D-Phe5 D-Trp7, 9 Leu11]-substance P (9 μM) plus ghrelin and LPS, and 4) an NF-κB inhibitor, parthenolide (10 μM) plus ghrelin and LPS.

Results—Ghrelin alone had no effect ($p>0.7$). Compared to LPS alone, ghrelin plus LPS decreased L_p (L_p : ghrelin+LPS=1.60±0.16 vs. LPS=2.27±0.14, $p<0.006$). The GHS-R1a ghrelin receptor antagonist blunted the effect of ghrelin by 86% during LPS-induced inflammation (L_p : ghrelin+LPS=1.60±0.16 vs. ghrelin antagonist+ghrelin+LPS=2.17±0.27, $p<0.018$). NF-κB inhibition did not influence the initial increased microvascular leak effect of ghrelin ($p>0.8$).

Conclusions—Although ghrelin has no effect on basal microvascular permeability, it has a biphasic effect with an overall decrease in microvascular permeability during LPS-induced inflammation through the GHS-R1a receptor, independent of NF-κB. Ghrelin is a key mediator of inflammation and may contribute to the increased morbidity and mortality in obese trauma patients.

INTRODUCTION

Obesity continues as a growing trend in the United States, with over 32% of the adult population with a BMI ≥30.[1] Moreover, unintentional injury remains the fifth leading cause of death among adults.[2] Obese trauma patients continue to be clinically challenging as they are more than five times more likely to die from their injury than their non-obese counterparts. In addition

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to other long term chronic health effects, obesity is an independent risk factor for increased morbidity and mortality after trauma.[3]

Ghrelin is a recently discovered hormone that is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R1a).[4] Produced mainly in the stomach, ghrelin was first described for its growth hormone releasing activity in the pituitary gland as well as its role in appetite stimulation through the activation of the hypothalamic neuropeptide Y-Y1 pathway.[5] The receptor for ghrelin is located in a variety of tissues, including lung, cardiomyocytes, intestine, and blood vessels.[6] Ghrelin has a variety of other growth hormone independent actions, including cardiomyocyte remodeling in rats with congestive heart failure (CHF) and inhibition of cardiomyocyte and endothelial cell apoptosis in vitro.[7] Recent studies also suggest that ghrelin may have anti-inflammatory and protective effects on endothelial cells.[8,9] The physiologic actions of ghrelin in down-regulating pro-inflammatory cytokines during sepsis may be in part due to stimulation of the vagus nerve and activation the cholinergic anti-inflammatory pathway.[10] However, the direct effect of ghrelin on endothelial microvascular permeability remains to be elucidated.

Loss of microvascular integrity after trauma contributes to the systemic inflammatory response syndrome, multiple organ failure, and significant morbidity and mortality.[11] The pathophysiology for differences in trauma outcomes of obese individuals is unclear; however it is known that circulating levels of ghrelin are decreased in patients with obesity.[12] Interestingly, ghrelin levels are negatively correlated with BMI; obese individuals have low levels of circulating ghrelin whereas individuals with anorexia nervosa have high levels of circulating ghrelin.[13] Moreover, in observational studies with rats, administration of ghrelin during septic shock decreases mortality and improves hypotension.[14] Thus, low levels of ghrelin may serve as a mechanistic link to explain the increased morbidity and mortality observed in obese trauma patients.

Our hypothesis was that ghrelin acts directly on endothelial cells to modify microvascular permeability. The purposes of this study were: 1) to determine the effect of ghrelin on basal state microvascular permeability, 2) to determine the effect of ghrelin on microvascular permeability during lipopolysaccharide (LPS)-induced inflammation, 3) to determine the involvement of the of the GHS-R1a endothelial cell receptor in the signal transduction mechanisms of ghrelin, and 4) to determine the involvement of the NF- κ B transcription factor in downstream signaling mechanisms of ghrelin.

MATERIALS AND METHODS

Animals

Institutional approval for this study was obtained and appropriate protocols for animal studies were followed. Adult female Sprague-Dawley rats (250g–310g; Hilltop Lab Animals Inc., Scottsdale, PA) were allowed free access to chow and water. Sodium pentobarbital (60 mg/kg body weight) was used to anesthetize the rats via subcutaneous injection. The small bowel mesentery was gently exposed via midline celiotomy and positioned over a quartz pillar for examination on an inverted microscope (Diaphot; Nikon) and continuously bathed in Ringer's solution. Mesenteric postcapillary venules, 20 to 30 μ m in diameter and at least 400 μ m in length, were identified based on flow patterns. Vessels with no evidence of leukocyte adherence or side branches were chosen for study.

Solutions and mediators

Ringer's solution was prepared with 135 mmol/L NaCl, 4.6 mmol/L KCl, 2.0 mmol/L CaCl₂, 2.46 mmol/L MgSO₄, 5.0 mmol/L NaHCO₃, 5.5 mmol/L dextrose, 11.04 mmol/L HEPES

acid, and 9.03 mmol/L HEPES salt (Research Organics, Cleveland OH). A 1% bovine serum albumin (BSA) solution was prepared before each experiment and added to all perfusion solutions (BSA crystallized; Sigma Chemical Co, St. Louis, MO).

The test perfusates consisted of adult female Sprague-Dawley rat red blood cell markers (250g–310g; Hilltop Lab Animals Inc., Scottsdale, PA), 1% BSA solution, and test mediator(s). The mediators included rat ghrelin (Phoenix Pharmaceuticals, Inc., Burlingame, CA), GHS-R1a receptor antagonist ([D-Arg1 D-Phe5 D-Trp7,9 Leu11]-substance P, Bachem, Torrance, CA), NF-κB inhibitor (parthenolide, Sigma, St. Louis, MO), and LPS (Sigma, St. Louis, MO).

Measurement of microvascular fluid leak

Hydraulic permeability (L_p), which is a measure of microvascular fluid leak, was measured in rat mesenteric post-capillary venules using an *in vivo* micro-occlusion technique. The assumptions and limitations of this method have been described in detail.[15] Briefly, single postcapillary mesenteric venules were cannulated with a micropipette containing Ringer's solution, marker red cells, and 1% BSA. A water manometer connected to the micropipette enabled manipulation of the hydrostatic pressure in the vessel. To measure transmural water flux (J_v), the vessel was occluded at a position distal to the cannulation site, permitting the intravascular pressure to equilibrate with the pressure in the micropipette as set with the water manometer. As fluid moves out of the vessel and into the interstitium, marker red cells advanced out of the micropipette and down the vessel toward the occluder.

Microvascular fluid leak (L_p) was determined on the basis of a modified version of Starling's equation for fluid filtration: $J_v = L_p * S[(P_c - P_i) - \sigma \Delta \Pi]$, where P_c is the capillary hydrostatic pressure, P_i is the interstitial hydrostatic pressure, σ is the osmotic reflection coefficient, and $\Delta \Pi$ is the colloid osmotic pressure gradient. Assuming the interstitial hydrostatic pressure (P_i) was near zero and the osmotic reflection coefficient (σ) and colloid osmotic pressure gradient ($\Delta \Pi$) remain constant, L_p was calculated from the slope of the regression of transmural water flux per unit surface area (J_v/S) on hydrostatic pressure (P_c) derived from several occlusions at three different hydrostatic pressures: $L_p = (J_v / S) / P_c$. A minimum of three different pressures (range 35 to 80 cm H₂O) were used for each estimate of L_p . Time studies have documented the stability of this model over time.[16]

Experimental design

For each study vessel, baseline L_p was first measured and recorded after perfusion with Ringer's/BSA solution for 10 minutes. Baseline L_p served as the control L_p for each study venule. Each vessel was then re-cannulated and perfused with the selected test mediator.

Effect of ghrelin on basal microvascular permeability

First, the effect of ghrelin on basal microvascular permeability was examined. Initial cannulation and baseline L_p measurements were performed, followed 10 minutes later by continuous perfusion of ghrelin (3μM). This dose was based on previously published data studying ghrelin levels during inflammation including data on systemic levels of ghrelin in rats during endotoxic shock which was measured at 29μM.[8,14,17–19] L_p was measured at initial perfusion with ghrelin, then at 5 minute intervals for a total of 30 minutes (n=5).

Effect of ghrelin on microvascular permeability during LPS-induced inflammation

The effect of ghrelin on microvascular permeability during LPS-induced inflammation was then assessed. Baseline L_p was measured, followed by systemic LPS administration via the rat femoral vein (10mg/kg) and L_p measured after 15 minutes. Local LPS (0.5mg/ml) and ghrelin

(3 μ M) were then continuously perfused into the study vessel, with L_p measured at 5 minute intervals for a total of 30 minutes (n=5).

Involvement of the of the GHS-R1a endothelial cell receptor in the signal transduction mechanisms of ghrelin

Involvement of the GHS-R1a endothelial cell receptor for ghrelin was examined by administration of the GHS-R1a ghrelin receptor antagonist during LPS-induced inflammation in the presence of ghrelin. Baseline L_p for the study vessel was measured. Next, continuous perfusion of [D-Arg1 D-Phe5 D-Trp7,9 Leu11]-substance P (9 μ M), a specific GHS-R1a ghrelin receptor antagonist, was administered for 30 minutes via the post-capillary venule. Continuous systemic LPS was then administered via (10mg/kg) and L_p measured after 15 minutes. Local LPS (0.5mg/ml), ghrelin (3 μ M), and [D-Arg1 D-Phe5 D-Trp7,9 Leu11]-substance P (9 μ M) were then continuously perfused into the study vessel, with L_p measured at 5 minute intervals for a total of 30 minutes (n=5).

Involvement of NF- κ B transcription factor in downstream signaling mechanisms of ghrelin

Involvement of the NF- κ B transcription factor in the ghrelin endothelial cell signaling pathway was examined by administration of parthenolide, a NF- κ B inhibitor, during LPS-induced inflammation in the presence of ghrelin. Baseline L_p for the study vessel was measured. Continuous systemic LPS (10mg/kg) was then administered via the rat femoral vein and L_p measured after 15 minutes. Next, local LPS (0.5mg/ml), ghrelin (3 μ M), and parthenolide (10 μ M) were then continuously perfused into the study vessel, with L_p measured at 5 minute intervals for a total of 30 minutes (n=3).

Statistical analysis

Differences within group means were analyzed by repeated measures ANOVA with post hoc analysis. Differences between group means were analyzed using ANOVA. Statistical significance was set at an alpha error of 5%. All values for L_p are reported as mean $\pm$ SEM $\times$ 10⁻⁷ cm $\times$ s⁻¹ $\times$ cm H₂O⁻¹.

RESULTS

Effect of ghrelin on basal microvascular permeability

Continuous ghrelin perfusion had no effect on basal microvascular permeability compared to baseline L_p (L_p ghrelin= 1.05 $\pm$ 0.03 vs. baseline=1.09 $\pm$ 0.05, $p>0.77$; figure 1).

Effect of ghrelin on microvascular permeability during LPS-induced inflammation

Compared to baseline, LPS alone increased microvascular permeability by 2-fold (L_p LPS = 2.07 $\pm$ 0.1, $p<0.02$). Interestingly, continuous perfusion of ghrelin during LPS-induced inflammation had a biphasic effect on microvascular permeability. Initially, at 5 minutes, ghrelin administration during LPS-induced inflammation increased microvascular leak from LPS alone (L_p ghrelin + LPS = 2.88 $\pm$ 0.23 vs. LPS= 2.07 $\pm$ 0.11, $p<0.001$); however by 20 minutes, a marked decrease in microvascular leak was observed with continuous perfusion of ghrelin during LPS-induced inflammation (L_p ghrelin + LPS = 1.60 $\pm$ 0.16 vs. LPS= 2.27 $\pm$ 0.14, $p<0.006$; figure 2). This effect continued to 30 minutes as ghrelin continued to decrease microvascular leak compared to LPS alone (L_p ghrelin + LPS = 1.25 $\pm$ 0.14 vs. LPS = 2.23 $\pm$ 0.11, $p<0.0001$; figure 2).

Involvement of the of the GHS-R1a endothelial cell receptor in the signal transduction mechanisms of ghrelin

Administration of the GHS-R1a endothelial cell receptor antagonist reduced the biphasic effect of ghrelin during LPS-induced inflammation. Initially at 5 minutes, microvascular leak with administration of the ghrelin receptor antagonist with ghrelin had no difference compared to ghrelin alone during LPS-induced inflammation (L_p ghrelin receptor antagonist + ghrelin + LPS = 2.88 ± 0.29 vs. ghrelin + LPS = 2.77 ± 0.24 , $p > 0.62$; figure 3). However, at twenty minutes, the decreased L_p seen with ghrelin during LPS-induced inflammation was blunted by 86% with administration of the ghrelin receptor antagonist (L_p ghrelin receptor antagonist + ghrelin + LPS = 2.17 ± 0.27 vs. ghrelin + LPS = 1.60 ± 0.16 , $p < 0.018$; figure 3). This trend continued to 30 minutes, with the GHS-R1a receptor antagonist continuing to blunt the effect of ghrelin during LPS-induced inflammation (L_p ghrelin receptor antagonist + ghrelin + LPS = 1.77 ± 0.13 vs. ghrelin + LPS = 1.25 ± 0.14 $p < 0.001$; figure 3).

Involvement of NF- κ B transcription factor in downstream signaling mechanisms of ghrelin

Administration of the NF- κ B inhibitor, parthenolide, did not influence the initial increase in microvascular leak observed with ghrelin during LPS-induced inflammation (L_p ghrelin + LPS = 2.88 ± 0.23 vs. ghrelin + parthenolide + LPS = 2.87 ± 0.11 , $p > 0.96$, figure 4). This suggests that the initial increase in microvascular leak does not involve NF- κ B activation by ghrelin. Moreover, at 20 minutes, no difference during LPS-induced inflammation in microvascular leak between ghrelin and the NF- κ B inhibitor plus ghrelin was observed (L_p ghrelin + LPS = 1.60 ± 0.16 vs. ghrelin + parthenolide + LPS = 1.76 ± 0.05 , $p > 0.52$, figure 4). This lack of difference in microvascular leak between ghrelin and the NF- κ B inhibitor plus ghrelin continued to 30 minutes (L_p ghrelin + LPS = 1.25 ± 0.14 vs. ghrelin + parthenolide + LPS = 1.28 ± 0.04 , $p > 0.83$, figure 4).

DISCUSSION

Despite similar age, gender, mechanism of injury, and injury severity score (ISS), when compared to non-obese trauma patients, obese trauma patients have increased complications, including increased incidence of multiple organ failure and acute respiratory distress syndrome. [20] The increased incidence of complications in obese trauma patients could be explained by low levels of circulating ghrelin. Ghrelin, a novel hormone that stimulates appetite, is paradoxically found at lower circulating levels in obese patients. Obese individuals have circulating ghrelin levels 32% lower than non-obese individuals.[12,21] The inverse relationship of weight and ghrelin levels is also observed in rats, the animal model used in this study.[22] In contrast, fasting plasma levels of ghrelin are elevated in patients with anorexia nervosa and return to normal levels after partial weight recovery.[23] This paradox of low levels of the hunger hormone ghrelin in obese individuals may reflect a negative feedback mechanism between ghrelin levels and adiposity as well as interaction with other related hormones, such as leptin.[13] We thought that because ghrelin has anti-inflammatory effects during inflammation, low levels of ghrelin in obese patients may in part explain the observed increase morbidity and mortality seen in obese trauma patients.

Ghrelin is thought to exert anti-inflammatory actions through activation of the cholinergic anti-inflammatory pathway via the vagus nerve, as well as through direct anti-inflammatory effects on neutrophils, lymphocytes, and macrophages.[10,24] Expression of ghrelin receptors on the vascular system is well-known and are thought to be upregulated in early sepsis.[17] However, the direct effects of ghrelin on endothelial cells during inflammation remains to be elucidated.

We hypothesized that ghrelin acts directly on endothelial cells to modify microvascular permeability. We found that although ghrelin had no effect on basal microvascular

permeability, during LPS-induced inflammation ghrelin overall decreases microvascular permeability and this effect is mediated via direct action on endothelial cells through the GHS-R1a receptor but not through downstream signaling of NF- κ B.

NF- κ B is transcription factor that upregulates expression of a variety of genes involved in the immune and inflammatory responses. We hypothesized that the initial increase in microvascular leak due to ghrelin during LPS-induced inflammation involved ghrelin's activation of NF- κ B through the direct interaction on endothelial cells. However, the NF- κ B inhibitor, parthenolide, did not affect the initial increase in microvascular leak due to ghrelin during LPS-induced inflammation. This suggests that the initial effect of ghrelin of increasing microvascular leak is independent of NF- κ B activation. Although TNF- α induced NF- κ B activation in endothelial cells is inhibited by ghrelin, other studies also suggest that ghrelin may act independently of NF- κ B during inflammation. [8,24]

The effect of ghrelin initially increasing microvascular permeability followed by a rapid decrease in microvascular permeability during inflammation was unexpected and may imply that ghrelin's effect may involve other inflammatory mediators that affect microvascular permeability. Wang and colleagues demonstrated that LPS decreases circulating levels of ghrelin and that a link between ghrelin and inflammatory mediators such as IL-1 and prostaglandins exists.[25] Also, ghrelin reduces IL-8 production in human umbilical vein endothelial cells (HUVECs) exposed to TNF- α , and also reduces release of the proinflammatory cytokines IL-1 β and IL-6.[8,26] Ghrelin may have an antagonistic role with leptin, a hormone that in addition to regulating satiety, metabolism, and energy expenditure, has been shown to be associated with levels of the inflammatory marker soluble vascular cellular adhesion molecule-1 (VCAM-1).[27] Interestingly, ghrelin inhibits leptin-induced proinflammatory cytokine expression by human monocytes and T cells.[26] This may partially explain the biphasic effect of ghrelin initially increasing microvascular permeability followed by a significant decrease in microvascular permeability during inflammation.

Because the micro-cannulation technique we use to measure microvascular permeability is a white blood cell-free system and isolates ghrelin's direct effect on the endothelium, our data suggests that ghrelin acts directly on endothelial cells. Further evidence that ghrelin acts directly on endothelial cells is provided by the GHS-R1a receptor antagonist data. The GHS-R1a receptor is specific for ghrelin; blockade of the ghrelin receptor in endothelial cells partially reversed ghrelin's decreased microvascular leak during LPS-induced inflammation. Li and colleagues noted a direct effect on endothelial cells and demonstrated that ghrelin inhibits TNF- α induced NF- κ B activation in endothelial cells.[8]

Ghrelin exhibits a biphasic effect during inflammation, with the ability to decrease microvascular leak. The initial increase in microvascular leak appears to be mediated through an NF- κ B independent mechanism. Our observation of ghrelin's ability to reverse LPS-induced increases in microvascular permeability may partially explain the increased morbidity and mortality in obese trauma patients. Obviously, clinical studies of ghrelin levels in obese and non-obese trauma patients are needed to explore ghrelin's role in possibly increasing morbidity and mortality in obese trauma patients.

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Figure 1. Effect of ghrelin on basal microvascular permeability

Ghrelin has no effect on basal microvascular fluid leak (L_p). Continuous perfusion of ghrelin alone at all time points did not significantly change L_p (L_p ghrelin = 1.05 ± 0.03 vs. baseline = 1.09 ± 0.05 , $p > 0.77$). Error bars denote $\pm$ SEM; L_p units are $\times 10^{-7}$ cm/sec/cmH₂O.

Figure 2. Ghrelin decreases microvascular permeability during LPS-induced inflammation

Continuous perfusion of ghrelin during LPS-induced inflammation had a biphasic effect on microvascular permeability. By 20 minutes, a marked decrease in microvascular leak was observed with continuous perfusion of ghrelin during LPS-induced inflammation (L_p ghrelin and LPS = 1.60 ± 0.16 vs. LPS = 2.27 ± 0.14 , $p < 0.006$), and continued to decrease microvascular leak to 30 minutes (L_p ghrelin and LPS = 1.25 ± 0.14 vs. LPS = 2.23 ± 0.11 , $p < 0.0001$). This data suggests that ghrelin reduces microvascular fluid leak during LPS-induced inflammation. Error bars denote $\pm$ SEM; L_p units are $\times 10^{-7}$ cm/sec/cmH₂O. * denotes statistically significant difference in microvascular leak of ghrelin and LPS compared to LPS alone.

Figure 3. Ghrelin receptor antagonist diminishes the effect of ghrelin on microvascular permeability during LPS-induced inflammation

The decreased L_p seen with ghrelin during LPS-induced inflammation was blunted by 86% with the administration of the ghrelin receptor antagonist within 20 minutes (L_p ghrelin receptor antagonist + ghrelin + LPS = 2.17 ± 0.27 vs. ghrelin + LPS = 1.60 ± 0.16 , $p < 0.018$). The blunting effect of the GHS-R1a receptor antagonist continued to 30 minutes (L_p ghrelin receptor antagonist + ghrelin + LPS = 1.77 ± 0.13 vs. ghrelin + LPS = 1.25 ± 0.14 , $p < 0.001$). This data suggests that ghrelin directly interacts with endothelial cells to reduce microvascular fluid leak during LPS-induced inflammation. Error bars denote $\pm$ SEM; L_p units are $\times 10^{-7}$ cm/sec/cmH₂O. *Significant L_p difference with ghrelin receptor antagonist perfusion along with ghrelin during LPS-induced inflammation versus ghrelin perfusion during LPS-induced inflammation.

Figure 4. Inhibition of NF-κB does not influence the initial increased microvascular leak effect of ghrelin during LPS-induced inflammation

Ghrelin alone during LPS-induced inflammation initially increased microvascular leak compared to LPS alone (L_p ghrelin + LPS = 2.88 ± 0.23 vs. LPS = 2.07 ± 0.11 , $p < 0.001$). Administration of parthenolide, an NF-κB inhibitor, did not affect the initial increased microvascular permeability effect of ghrelin during inflammation (L_p ghrelin + LPS = 2.88 ± 0.23 vs. ghrelin + parthenolide + LPS = 2.87 ± 0.11 , $p > 0.96$). At 20, 25, and 30 minutes, no significant difference was seen between ghrelin and the NF-κB inhibitor plus ghrelin during LPS-induced inflammation. Error bars denote $\pm$ SEM; L_p units are $\times 10^{-7}$ cm/sec/cmH₂₀.