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Perirenal fat promotes renal arterial endothelial dysfunction in obese swine through tumor necrosis factor- α

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Abstract

Purpose—Perirenal fat is associated with poor blood pressure control and chronic kidney disease, but the underlying mechanisms remain elusive. We tested the hypothesis that perirenal fat impairs renal arterial endothelial function in pigs with obesity-metabolic derangements (ObM).

Material and Methods—Fourteen domestic pigs were studied after 16 weeks of a high-fat/high-fructose diet (ObM) or standard chow (Lean). Renal blood flow (RBF), glomerular filtration rate (GFR), and visceral fat volumes were studied *in-vivo* with CT. Renal arterial endothelial function was also studied *ex-vivo* in the organ bath.

Results—ObM pigs demonstrated increased body weight, blood pressure, cholesterol, and intra-abdominal fat compared to lean pigs, and perirenal fat volume was significantly larger. RBF and GFR were markedly elevated, while urinary protein level was preserved. *Ex-vivo* acetylcholine-induced endothelium-dependent vasodilation of renal artery rings was substantially impaired in ObM compared to Lean. Endothelial function was further blunted in both ObM and Lean arterial rings by incubation with perirenal fat harvested from ObM, but not from Lean pigs, and was restored by inhibition of tumor necrosis factor (TNF)- α . ObM perirenal fat also showed increased pro-inflammatory macrophage infiltration and TNF- α expression.

Conclusions—ObM perirenal fat directly causes renal artery endothelial dysfunction, partly mediated by TNF- α .

Keywords

obesity; adipose tissue; chronic kidney disease

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

INTRODUCTION

Metabolic syndrome is a cluster of metabolic disorders partially attributed to intra-abdominal fat deposition.¹ Ectopic adipose tissue, especially abdominal visceral adipose tissue, contributes to the association of adiposity with cardiometabolic risk.² The adipose tissue surrounding the kidney, including perirenal and renal sinus fat, is not only an energy storing but also a metabolically active tissue, regulating the physiology and pathophysiology of adjacent organs.³ Indeed, the perirenal fat thickness has emerged as an independent predictor of renal dysfunction in patients with diabetes,⁴ and renal sinus fat has been associated with poor blood pressure control.⁵ However, while perirenal fat may be associated with renal vascular or parenchymal disorders,⁶ its precise role remains unknown.

Dysfunction of the renal arterial endothelium predicts development of renal damage in several animal models.^{7, 8} Vascular endothelial dysfunction can be substantially altered by adjacent adipose tissue, especially perivascular fat.⁹ Ectopic fat in obesity is characterized by elevated expression of tumor necrosis factor (TNF)- α , which contributes to endothelial dysfunction in resistance arteries embedded within epididymal fat.¹⁰ Kidney-derived TNF- α also promotes angiotensin II-induced hypertension.¹¹ Yet, the potential paracrine effect of perirenal fat TNF- α on endothelial function of the adjacent renal arteries remains unknown. Therefore, this study was designed to test the hypothesis that perirenal fat in pigs with obesity-metabolic derangement (ObM) directly impairs endothelial function of the renal artery, and that this effect is partly mediated by TNF- α .

MATERIAL AND METHODS

Animal protocols

This study was approved by the Mayo Clinic Institutional Animal Care and Use Committee. Fourteen 3-month-old female domestic pigs were randomized into two groups of ObM and lean controls (n=7 each). Lean pigs were fed regular chow, while ObM pigs started a high-fat/high-fructose diet (5B4L; Purina Test Diet, Richmond, Indiana) that contains (in % kcal) 17% protein, 20% complex carbohydrates, 20% fructose, and 43% fat (lard, hydrogenated soybean, and coconut oils). At 16 weeks, pigs were anesthetized with 0.5g of intramuscular ketamine and xylazine, and anesthesia was maintained with intravenous ketamine (0.2 mg/kg/min) and xylazine (0.03 mg/kg/min). Arterial blood pressure was monitored using an intra-arterial catheter, and an intravenous glucose-tolerance test was performed. Then, hemodynamics, function, and visceral fat volumes in each kidney were assessed using multidetector computed tomography (MDCT), and renal venous and arterial blood collected, as were urine samples. The animals were then allowed a 3-day recovery period, and subsequently euthanized with a lethal intravenous dose of pentobarbital (Fatal-Plus®, Vortech Pharmaceuticals, Dearborn, MI, 100mg/kg). The kidneys and perirenal fats were removed using a retroperitoneal incision and immediately dissected, and sections were frozen in liquid nitrogen and maintained at -80°C. The renal arteries and a small piece of perirenal fat were collected immediately for *ex-vivo* organ bath experiments.

MDCT

At 16 weeks, MDCT scanning was performed to assess kidney volume, renal blood flow (RBF) and glomerular filtration rate (GFR), as described previously.¹² Briefly, 160 consecutive scans were performed following a central venous injection of iopamidol ($0.5 \text{ ml} \cdot \text{kg}^{-1} \cdot 2\text{s}^{-1}$). Then, the same procedure was repeated after a 15-min interval and toward the end of a 10-min intra-aortic infusion of acetylcholine (ACh; $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) into a tracker catheter placed above the renal arteries to test endothelium-dependent microvascular reactivity *in-vivo*. MDCT images were reconstructed and displayed with the ANALYZE™ software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). RBF and GFR were measured from tissue time-attenuation curves obtained in regions of interest selected from the aorta, renal cortex and medulla. Renal vascular resistance was calculated by dividing mean arterial pressure by RBF. Intra-abdominal adipose tissue was measured and expressed as volume and fraction, and perirenal fat volume as ratio to the volume of the corresponding kidney, as described previously.¹³

Organ bath experiments

Renal arteries were dissected and cut into 5-mm-long segments in cold Krebs solution containing (in mmol/L): NaCl 119; NaHCO₃ 25; KCl 4.7; KH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 2.5, and Glucose 11.1; pH 7.4. The renal artery rings were mounted between 2 hooks attached to an isometric force transducer with continuous recording of tension and were immersed in organ chambers containing 10mL of Krebs solution gassed with 95% O₂ and 5% CO₂ at 37°C. The resting tension was adjusted to 2g, and the bath solution replaced every 15 min. After a 60-min equilibration period, the viability of each ring was tested by exposure to a high-K⁺ Krebs solution (60 mmol/L KCl), and rings were allowed to rest in the organ bath for 30 min before any drugs were administered. The integrity of the endothelium was assessed by relaxation to 10^{-6} mol/L ACh. Incremental doses of phenylephrine (10^{-9} – 10^{-5} mol/L) were used to test contractile response. For measuring the relaxation response, a sustained contraction was induced by adding $3 \mu\text{mol/L}$ phenylephrine. Relaxations caused by incremental doses of ACh (10^{-9} – 10^{-4} mol/L) and sodium nitroprusside (SNP, 10^{-9} – 10^{-4} mol/L) were recorded. A group of rings were incubated for 30 minutes in 10mL of Krebs solution in the absence or presence of fresh perirenal fat (100g) obtained from allogeneic ObM or Lean pigs, with or without co-incubation with neutralizing anti-TNF- α antibodies (20 $\mu\text{g/mL}$), the superoxide radical scavenger tempol (1 mmol/L), or the thromboxane A₂ (TXA₂) synthase inhibitor dazoxiben (1 mmol/L). Vasorelaxant responses are expressed as percentage reduction in tone induced by phenylephrine. Vasoconstriction responses are expressed as percentage contraction in tone induced by 60 mmol/L KCl.

Blood and urine assays

Before MDCT scanning, an intravenous glucose-tolerance test was performed and the homeostasis model assessment insulin resistance index (HOMA-IR, fasting plasma glucose $\times$ fasting plasma insulin/22.5) used as an index of insulin resistance. Urine was collected by bladder puncture and blood samples from the inferior vena cava and renal artery. Urine microalbumin (Arbor Assays, Ann Arbor, MI), serum creatinine, and lipid profile (Roche)

including triglycerides, total cholesterol, low-density lipid-cholesterol (LDL-C) and high density lipid-cholesterol (HDL-C) were assessed. Renal venous and arterial plasma levels of inflammatory cytokines were measured by Luminex (Millipore), including TNF- α , interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), and interferon- γ (IFN- γ). The cross-kidney gradients of renal venous and arterial levels of these cytokines between were calculated, and their net release from the kidney computed as [(venous–arterial levels) X RBF].

Tissue inflammation

Macrophage (M ϕ) infiltration in perirenal fat was assessed by immunofluorescence staining of M ϕ marker CD68 (1:50, Abcam), and their subpopulations by coexpression of either inducible nitric oxide synthase (1:50, Abcam) for pro-inflammatory M1-M ϕ or arginase-1 (1:50, Abcam) for reparative M2-M ϕ . Positive and double-positive cells were counted. TNF- α and IL-6 immunoreactivity in perirenal fat tissue were examined using specific antibodies (1:50, Abcam), and their mRNA expression by qPCR. TNF- α and IL-6 immunoreactivity was calculated using a computer-aided image-analysis program. In each slide immunostaining was quantified in 15–20 fields, expressed as % of staining of total surface area, and the results from all fields averaged.

Oxidative stress

The *in situ* production of superoxide anion in perirenal fat tissues was evaluated by fluorescence microscopy after dihydroethidium (DHE) staining. The ratio of staining-positive area (red) and nuclear area (blue) was calculated.

Statistical analysis

Results are expressed as mean $\pm$ SE. Statistical analysis was performed using JMP software package version 8.0 (SAS Institute, Cary, NC). Comparisons between groups were performed using unpaired *t*-test, and within groups using paired *t*-test and repeated measures ANOVA. Statistical significance for all tests was accepted for $P<0.05$.

RESULTS

Animal characteristics

After 16 weeks of diet, the pigs in ObM group had higher body weight (Figure 1A, $P<0.01$), systolic blood pressure (Figure 1B, $P<0.01$), HOMA-IR index (Figure 1C, $P<0.05$), and cholesterol levels (Figure 1D, all $P<0.01$). Bilateral perirenal fat tissues, reflected by the ratio of perirenal fat-to-kidney volumes, were both markedly higher in the ObM compared to the lean group (Figure 1E and F, both $P<0.01$). Additionally, the intra-abdominal fat volume fraction of ObM pigs was greater than the lean group (Figure 1G, $P<0.01$). Collectively, ObM pigs showed typical characteristics of obesity-metabolic syndrome, with a cluster of metabolic disorders including overweight, visceral adiposity, hypertension, insulin resistance, and dyslipidemia.

Renal hemodynamics and function

Baseline and Ach-induced RBF were both significantly higher in ObM group than lean group (Figure 1H, both $P<0.01$). Ach increased RBF similarly in both ObM (+34%, $P<0.05$) and lean pigs (+35%, $P<0.01$) compared with baseline. Renal vascular resistance was significantly lower in ObM compared to lean pigs both before and after Ach injection (Figure 1I, both $P<0.05$), and fell in both groups in response to Ach. GFR was markedly increased (Figure 1J, $P<0.01$), while plasma creatinine level was decreased (Figure 1K, $P<0.05$), probably secondary to glomerular hyperfiltration.¹⁴ Urinary protein level was unchanged in ObM compared to the lean group (data not shown).

Renal arterial endothelial function *ex-vivo*

Ach-induced endothelium-dependent relaxation of the renal artery *ex-vivo* was significantly impaired in ObM compared to lean pigs (Figure 2A), while SNP-induced endothelium-independent relaxation and Phe-induced vasoconstriction were similar between these two groups (Figure 2B and C). Ach-induced relaxation of renal arteries obtained from lean pigs was unaffected by a 30-min incubation with perirenal fat obtained from lean pigs (Figure 2D). In contrast, it was significantly attenuated after incubation with perirenal fat obtained from ObM pigs (Figure 2D). Similarly, Ach-induced relaxation of the ObM renal artery was not influenced by incubation with perirenal fat from lean pigs, but was impaired by incubation with perirenal fat from ObM pigs (Figure 2E). Contrarily, SNP-induced relaxation of both lean and ObM renal arteries was not affected by perirenal fat from either lean or ObM pigs (Figure 2F and G).

Importantly, co-incubation of ObM rings with neutralizing anti-TNF- α antibodies, but not with the antioxidant tempol or the TXA2 synthase inhibitor dazoxiben, normalized endothelial function of the renal artery that was impaired by ObM perirenal fat (Figure 2H).

Perirenal fat inflammation and oxidative stress

The infiltration of pro-inflammatory M1-M ϕ ($P<0.01$), but not anti-inflammatory M2-M ϕ , increased in ObM compared with Lean perirenal fat (Figure 3A–C). The immunoreactivity (Figure 3D–F) and mRNA expression (Figure 3G and H) of TNF- α , but not IL-6, were several fold higher in ObM perirenal fat. Contrarily, neither renal arterial blood levels, renal venous blood levels, nor kidney release of the inflammatory markers TNF- α , IL-6, MCP-1, and IFN- γ , were increased in the ObM group (Table 1).

DHE fluorescent staining was increased in perirenal fat from ObM group compared with lean group ($P<0.01$, Figure 4A and B), suggesting increased *in-situ* superoxide production.

DISCUSSION

This study shows that ObM pigs have expansive perirenal fat with increased M1-M ϕ infiltration, TNF- α expression, and oxidative stress compared with lean pigs. Moreover, perirenal fat of ObM, but not of lean pigs, directly impairs renal arterial endothelial function, which can be improved by neutralization of TNF- α . These observations suggest that

perirenal fat exerts paracrine effects on the renal circulation in obesity, and implicate fat-produced TNF- α in these effects.

Our previous studies have demonstrated that swine ObM induces renal hyperperfusion and glomerular hyperfiltration.¹⁵ These may result from increased cardiac output and hyperinsulinemia, and have been viewed as early pathogenic events in the development of chronic kidney disease (CKD).¹⁶ This study shows that despite the increased RBF and glomerular hyperfiltration in ObM pigs, endothelial function of their renal arteries is significantly impaired. Endothelial dysfunction is a predictor of renal vascular and parenchymal diseases.^{17–19} However, the mechanism underlying the ObM-associated renal artery endothelial dysfunction remained largely unknown.

The adipose tissue is no longer considered a passive energy storage tissue, but an active paracrine and endocrine tissue. Ectopic fat plays a critical role in physiological and pathophysiological regulation of its adjacent organ.²⁰ In particular, the epicardial and coronary perivascular fat is linked to development of cardiovascular disorders.^{21, 22} The adipose tissues surrounding the kidney, including perirenal and renal sinus fat, have received increased attention during recent years. The amount of perirenal fat shows a good correlation with central obesity,²³ poor blood pressure control, and development of CKD.^{4, 5} However, the causal relationship between perirenal fat and renal vascular and parenchymal disorders is yet to be established.

The adipose tissue imparts both protective and deleterious effects on vascular function of adjacent arteries.²⁴ In lean healthy subjects, perivascular fat exerts on adjacent arteries an anti-contractile effect, which is lost in obesity, metabolic syndrome, and diabetes.²⁵ Coronary perivascular adipose tissue from obese Ossabaw pigs in fact potentiates contractions of their coronary vascular smooth muscle.²² Similarly, our study shows that perirenal fat in ObM, but not of lean pigs, impairs endothelial function of the renal artery, while endothelium-independent relaxation remains intact, suggesting preserved vascular smooth muscle cell responses. The differential effect of ObM fat may be due to fat inflammation and increased oxidative stress elicited by metabolic derangements. Indeed, compared with lean pigs, we found in perirenal fat from ObM pigs increased pro-inflammatory M1-MØ infiltration, enhanced TNF- α protein and mRNA expression (suggesting local production), and elevated *in-situ* superoxide production, indicating a local inflammatory and oxidative state. As observed in many fat depots,²⁶ macrophages infiltrating perirenal fat early during evolution of obesity likely release TNF- α , which in turn causes inflammation and oxidative stress, both of which can induce endothelial dysfunction. Notably, neutralization of TNF- α attenuated perirenal fat-induced endothelial dysfunction of the renal artery *ex vivo*, whereas neither the antioxidant tempol nor a TXA2 synthase inhibitor did. We interpret these results to suggest that in our model inflammation plays a greater role in mediating fat-induced endothelial dysfunction than do superoxide anion or the vasoconstrictor TXA2. Indeed, TNF- α exerts pleiotropic effects on the vasculature, including downregulation of endothelial nitric oxide synthase expression and limiting nitric oxide bioavailability.¹¹ Several previous studies demonstrated that intravascular administration of TNF- α -neutralizing antibody infliximab reduces blood pressure and ameliorates endothelial function in patients with rheumatoid arthritis.^{27, 28} The renal arterial

blood levels of inflammatory markers were not increased in ObM pigs, arguing against their endocrine effects on endothelial function of the renal artery. Similarly, the unchanged renal venous blood levels or release of these inflammatory markers in ObM compared to lean pigs suggests that kidney production is unlikely the source of TNF- α impairing the function of the renal artery. These observations support the contention that perirenal fat-derived TNF- α may affect the renal artery in a paracrine manner.

Interestingly, RBF and renal vascular resistance responses to Ach *in-vivo*, which reflect microvascular endothelial function, were relatively preserved in ObM kidneys, and might initially partly compensate for the impaired reactivity of large renal arteries. Notably, renal vascular resistance is regulated chiefly by intrarenal microvessels, and may thus compensate for abnormal renal arterial vasoreactivity. Nonetheless, blunted vasorelaxation represents a variety of endothelial functions that might be impaired in ObM, such as anti-inflammatory and anti-thrombogenic activities, which may still impact the renal artery locally. A similar dissociation between coronary epicardial and microvascular endothelial function can be observed in patients with early atherosclerosis.²⁹ However, microvascular dysfunction may well fall in more advanced or prolonged ObM.

The major limitation of this study is the challenge in demonstrating the direct effect of perirenal fat on the renal artery *in vivo*. While perirenal fat may damage renal vascular endothelial function through TNF- α , the precise paracrine mechanism also warrants further studies. The mediators of Ach-induced dilation were not further identified due to the limited renal arterial samples, and additional mechanisms may contribute to endothelial dysfunction in this model. In addition, given that adipocyte size increases in obesity,³⁰ the same mass of perirenal fat used to treat renal arteries *ex vivo* might have contained different tissue proportions of adipocytes, vessels, and interstitial cells in lean versus ObM pigs. Furthermore, our pigs were exposed to a 16-week diet; longer duration in human subjects may accelerate deterioration of renal function.

CONCLUSIONS

In conclusion, the perirenal fat under ObM conditions shows increased volume, inflammation, and oxidative stress, and causes endothelial dysfunction of renal arteries, likely mediated in a paracrine fashion by TNF- α . Blockage of TNF- α may prove to be an important strategy to combat ObM related renal endothelial dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS AND ACRONYMS

Ach	acetylcholine
CKD	chronic kidney disease
DHE	dihydroethidium
GFR	glomerular filtration rate
HDL-C	high density lipid-cholesterol
IFN-γ	interferon- γ
IL-6	interlukin-6 (IL-6)
LDL-C	low-density lipid-cholesterol
MCP-1	monocyte chemoattractant protein-1
MDCT	multidetector computed tomography
MØ	macrophage
ObM	obesity-metabolic derangement
Phe	phenylephrine
RBF	renal blood flow
SNP	sodium nitroprusside
TNF-α	tumor necrosis factor- α
TXA2	thromboxane A2

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Figure 1.

Characteristics of ObM pigs. Body weight (A), systolic blood pressure (BP) (B), HOMA-IR index (C), plasma total cholesterol (TC), LDL-C, and HDL-C (D) of ObM pigs (black bars or symbols) were all elevated compared to lean pigs (white bars or symbols) after a 16-week diet, whereas triglycerides (TG) levels (D) were similar between these two groups. (E) Representative computed tomography. White arrows indicate perirenal fat (yellow). Perirenal fat (F) and intra-abdominal fat (G) of ObM pigs (black bars) were increased compared to lean pigs (white bars). At baseline and in response to acetylcholine, renal blood flow (H) was higher while renal vascular resistance (I) was lower in ObM pigs (black bars) than lean pigs (white bars). Glomerular filtration rate (J) was increased while plasma creatinine (K) level was decreased in ObM pigs (black bars) compared to lean pigs (white bars). Data are expressed as means $\pm$ SE, n=7 each group. * P <0.05, ** P <0.01 vs. lean group; # P <0.05, ## P <0.01 vs. baseline.

Figure 2.

Endothelial function of renal arteries. Ach- (A) and SNP- (B) induced relaxation and phenylephrine (Phe)-induced constriction (C) of renal arteries from lean and ObM pigs. * $P < 0.05$, ** $P < 0.01$ vs. lean group. (D) Ach-induced relaxation of lean pigs' renal arteries incubated with or without perirenal fat from lean and ObM pigs. * $P < 0.05$, ** $P < 0.01$ vs. lean group. (E) Ach-induced relaxation of ObM pigs' renal arteries treated with or without perirenal fats from lean and ObM pigs. * $P < 0.05$, ** $P < 0.01$ vs. ObM group. (F) SNP-induced vasodilation of lean pigs' renal arteries incubated with or without perirenal fat from

lean and ObM pigs. **(G)** SNP-induced relaxation of ObM pigs' renal arteries treated with or without perirenal fats from lean and ObM pigs. **(H)** Ach-induced relaxation of ObM pigs' renal arteries incubated with perirenal fat from ObM pigs in the present or absent of anti-TNF- α neutralizing antibodies, antioxidant tempol, or thromboxane A2 synthase inhibitor dazoxiben. * $P<0.05$ vs. ObM+ObM fat group. Data are expressed as means $\pm$ SE, n=5–8 arterial rings from 3–5 pigs.

Figure 3.

Perirenal fat inflammation. (A) Representative images (40×) of immunofluorescence staining for M1- and M2- MØ (CD68: red; M1, inducible nitric oxide synthase or M2, arginase-1: green; double staining: yellow) in perirenal fat from lean and ObM pigs. (B and C) Quantification of M1-MØ- and M2- MØ-positive cells. (D) Representative images (40×) of immunofluorescence staining for TNF-α (green) and IL-6 (red) in perirenal fat from lean and ObM pigs. (E and F) Quantification of TNF-α- and IL-6-positive cells. The mRNA expression of TNF-α (G) and IL-6 (H) in perirenal fat from lean and ObM pigs. (Data are expressed as means±SE, n=7 each group. * $P<0.05$, ** $P<0.01$ vs. lean group.

Figure 4.

Perirenal fat oxidative stress. **(A)** Representative images (40×) of staining for superoxide anion with DHE fluorescent dye in perirenal fat from lean and ObM pigs. DHE: red; DAPI: blue. **(B)** Quantification of DHE/DAPI-positive area. Data are expressed as means±SE, n=7 each group. * $P<0.05$ vs. lean group.

Table 1

Renal arterial and venous blood levels and kidney release of inflammatory cytokines in lean and ObM pigs.

	Cytokines	Lean	ObM	<i>P</i> -value
Renal arterial blood	TNF-α, pg/mL	24.04 $\pm$ 4.92	35.04 $\pm$ 11.31	0.19
	IL-6, ng/mL	0.96 $\pm$ 0.09	1.10 $\pm$ 0.10	0.17
	MCP-1, ng/mL	0.15 $\pm$ 0.02	0.18 $\pm$ 0.02	0.18
	IFN-γ, ng/mL	0.16 $\pm$ 0.06	0.23 $\pm$ 0.13	0.31
Renal venous blood	TNF-α, pg/mL	23.72 $\pm$ 5.26	37.11 $\pm$ 14.46	0.20
	IL-6, ng/mL	0.98 $\pm$ 0.11	1.15 $\pm$ 0.86	0.13
	MCP-1, ng/mL	0.12 $\pm$ 0.01	0.16 $\pm$ 0.03	0.11
	IFN-γ, ng/mL	0.15 $\pm$ 0.07	0.22 $\pm$ 0.13	0.33
Kidney release	TNF-α, ng/min	-0.19 $\pm$ 0.30	1.40 $\pm$ 2.42	0.26
	IL-6, ng/min	12.65 $\pm$ 21.05	43.67 $\pm$ 27.54	0.19
	MCP-1, ng/min	-19.36 $\pm$ 9.05	-16.50 $\pm$ 17.50	0.44
	IFN-γ, ng/min	-8.25 $\pm$ 14.96	-19.20 $\pm$ 10.43	0.30

Data are mean $\pm$ SE. n=7 each group.