Rosiglitazone protects against ischemia/reperfusion-induced leukocyte adhesion in the Zucker diabetic fatty rat.


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Running title: Rosiglitazone inhibits leukocyte adhesion in ZDF rats \textit{in vivo}.

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Non-standard abbreviations: RSG, rosiglitazone maleate; PPAR-\(\gamma\), peroxisome proliferator-activated receptor-\(\gamma\); VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1
ABSTRACT

Increased susceptibility to atherosclerosis increases the risk of mortality in type 2 diabetic patients. Leukocyte adhesion to the endothelium is a critical step in atherogenesis. In addition to its insulin-sensitizing effects, rosiglitazone (RSG) possesses anti-inflammatory properties. The effects of RSG, however, on the initial phase of leukocyte recruitment (rolling, adhesion) have not been studied in vivo. This study tested the hypothesis that RSG treatment of Zucker diabetic fatty (ZDF) rats inhibits ischemia-reperfusion-induced leukocyte adhesion to the endothelium. Male ZDF rats (16 wk) were treated with RSG (3mg/kg/d, p.o.) 7d before experimentation. Leukocyte-endothelial interactions in cremaster venules were recorded using intravital microscopy prior to 30min of ischemia and during a 90min reperfusion period. While blood pressure, plasma glucose and insulin were not different between treatment groups, RSG treatment was associated with reduced leukocyte rolling and inhibition of leukocyte adhesion throughout the reperfusion period (P<0.01). Cremaster mRNA expression of VCAM-1 was reduced by 35% in RSG-treated animals (P<0.01), whereas P- and E-selectin and ICAM-1 were unchanged. Immunostaining for P-selectin, E-selectin, and VCAM-1 was reduced by 21%, 61%, and 50%, respectively (for all, P<0.05) in RSG-treated animals. Inhibition of ischemia/reperfusion-induced leukocyte adhesion might contribute to the utility of RSG as a therapy for vascular disease.
INTRODUCTION

Cardiovascular disease is a major cause of mortality in type 2 diabetic patients (Hurst and Lee 2003). Obesity, dyslipidemia, hypertension, insulin resistance/glucose intolerance, as well as a proinflammatory and prothrombotic state, characteristics of Metabolic Syndrome (Grundy et al., 2004), are thought to contribute to the accelerated atherosclerosis (Kannel and McGee, 1979; Uusitupa et al., 1993) and post-myocardial infarction mortality seen in type 2 diabetics (Woodfield et al., 1996; Kereiakes 1985). One of the potential mechanisms which may contribute to vascular disease these patients is endothelial dysfunction, evidenced by impaired endothelium-dependent vasodilation and augmented responses to vasoconstrictors in preclinical models of Metabolic Syndrome (Walker et al., 1997; Walker et al. 1999). Increased leukocyte adhesion to the endothelium is seen in response to ischemia/reperfusion injury (Lefer, 1995; del Zoppo and Garcia, 1995) and is widely recognized as a critical step in the initiation of atherosclerosis (Ross, 1999).

Rosiglitazone (RSG), a member of the thiazolidinedione family of peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, is currently used for the treatment of type II diabetes mellitus. The insulin-sensitizing and glucose-lowering effects of thiazolidinediones such as RSG in preclinical animal models (Fujiwara et al. 1998; Oakes et al., 1994) and in human patients (Wagstaff and Goa, 2002) are well-documented. In addition, thiazolidinediones have been shown to inhibit adhesion molecule expression in vitro (Pasceri et al., 2000; Ricote et al., 1998), and to inhibit other inflammatory processes such as cytokine release (Boyle, 2004). Indeed, RSG and other thiazolidinediones have been shown to reduce cardiovascular risk factors associated with atherosclerosis (Roberts et al., 2003), though the effects of these agents on leukocyte recruitment have been studied only indirectly. Acute RSG treatment has been shown
histologically to reduce the presence of leukocytes in ischemia-reperfused myocardium (Yue et al., 2001), however the exact stage of leukocyte recruitment at which RSG exerts its effects has not been determined. Furthermore, the effects of RSG on ischemia/reperfusion-induced leukocyte adhesion have not been investigated in vivo. Therefore, the purpose of the present study was to test the hypothesis that acute RSG treatment inhibits ischemia/reperfusion-induced leukocyte adhesion in the ZDF rat.
METHODS

Animals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the GlaxoSmithKline Animal Care and Use Committee. Male Zucker diabetic fatty (ZDF) rats (16-18 wk old) were obtained from Charles River (Wilmington, MA). Rosiglitazone (3 mg/kg/d) or vehicle (0.9% saline, 1% DMSO v/v) was administered via oral gavage once daily for 7 d prior to experimentation (n=10 rats per treatment group). This dose was previously shown to protect against myocardial infarction in normal rats (Yue et al., 2001). Rats were fasted overnight prior to experimentation.

Experimental preparation

On the day of experimentation, rats were anesthetized with isoflurane (2.5% ) the right femoral artery was cannulated for direct blood pressure recording (Ponemah Physiology Platform, Gould Instrument Systems, Inc., Valley View, OH). Isoflurane was reduced to 1.5% and rats allowed to equilibrate for 10 min. Pressure data was recorded every 10s for 5 min. The cremaster muscle was prepared as recently described (Johns et al., 2005).

Intravital microscopy

Intravital microscopy was performed using an upright water immersion microscope (Olympus BX51WI, B&B Microscopes, Warrendale, PA). A 4X objective lens was used to select between 3 and 6 venules with consistent blood flow characteristics from each cremaster preparation. For each venule, centerline red blood cell velocity was measured using an optical doppler and cross-correlation system (CircuSoft, Hockessin, DE) and shear rate was calculated as described (Johns et al., 2005).

Experimental procedures
Images of cremaster venules were acquired with a high-speed digital video camera (Sensicam VGA, Cooke Corp., Londonderry, NH) coupled to MetaMorph™ imaging software (Universal Imaging Corporation, Downingtown, PA). A single bright-field image (20X) was taken of each venule for venule diameter and length measurement. Leukocytes were fluorescently labeled (Rhodamine 6G, 0.3mg/kg i.v. bolus) prior to experimentation via a right femoral vein cannula. Labeled leukocytes were epi-illuminated with a mercury arc lamp, 20X water immersion objective lens and rhodamine filter cube. Timelapse image stacks (1 frame/s for 30s) and streaming video (66.67 frames/s for 4.5s) were generated for each venule prior to ischemia for determination of baseline leukocyte adhesion/rolling flux and leukocyte rolling velocity, respectively.

Following baseline measurements, ischemia was induced by placing two vascular clamps on the cremaster tissue at its entry point at the abdominal cavity. Cessation of blood flow was verified in each vessel visually. In pilot experiments, 30min of ischemia was found to produce a reproducible increase in leukocyte adhesion without sustained reductions in post-reperfusion blood flow as seen in longer ischemia periods. Following 30min of ischemia, clamps were removed and the return of blood flow verified via microscopic observation. Each venule was recorded at 5, 15, 30, 45, 60 and 90min post-ischemia.

Following the 90min time point, rats were euthanized while under isoflurane anesthesia by cervical dislocation and exsanguination. Blood was collected for white cell count and plasma endpoint measurements, and cremaster, aorta, abdominal and perivascular adipose tissues were removed, flash-frozen in liquid nitrogen, and stored at -80°C for later use in immunohistochemistry and RNA isolation for real-time quantitative PCR.

Image data analysis
The number of adherent cells was defined as the number of cells that did not move during the timelapse period (30s) per 100\(\mu\)m of venule length (Gavins and Chatterjee, 2004), normalized to white cell count. Rolling leukocyte flux was defined as the number of cells rolling past a fixed line perpendicular to the direction of blood flow during the streaming video period, normalized to white cell count (Gavins and Chatterjee, 2004). Rolling cells were designated as those which were moving slower than the centerline blood flow (Gavins and Chatterjee, 2004). For each animal, the rolling velocities of ~5 leukocytes per venule were determined at each time point using MetaMorph object tracking.

**Plasma measurements**

Plasma insulin and adiponectin were determined by Linco diagnostics (St. Charles, MO) using an enzyme-linked immunosorbent assay. Plasma glucose and free fatty acids were determined as previously described (Jucker et al., 2005). White blood cell counts were determined with the Advia 120 instrument, according to the manufacturer’s instructions (Bayer Diagnostics, Tarrytown, NY).

**Real-time quantitative PCR**

Cremaster tissues were homogenized in liquid nitrogen using a mortar and pestle and total RNA extracted using RNAzol, according to manufacturer's instructions (GIBCO BRL, Gaithersburg, MD). Total RNA was extracted from tissues using Qiagen RNeasy Maxi kit (Qiagen, Inc., Santa Clarita, CA). Primers and TaqMan probes for VCAM-1, ICAM-1, P-selectin, E-selectin, MCP-1, and the housekeeping gene RPL32 were designed using Primer Express software (Foster City, CA). Primer sequences can be found in Table 1. All TaqMan probes were labeled with FAM and TAMRA as the reporter and quencher dyes, respectively. Quantitative RT-PCR was
performed using the ABI Prism 7700 system (Applied Biosystems) as previously described (Johns et al., 2004).

**Immunohistochemistry**

Immunohistochemistry for adhesion molecules was performed using the following primary antibodies: goat polyclonal anti-rat VCAM-1 (1:50), goat polyclonal anti-mouse P-Selectin (3\(\mu\)g/ml), goat polyclonal anti-rat-E-Selectin (15\(\mu\)g/ml) (all from R&D Systems, Minneapolis, MN), mouse monoclonal anti-rat ICAM-1 (1\(\mu\)g/ml) (BD Pharmingen, San Diego, CA). Sections treated with the goat polyclonal primary antibodies were then incubated with biotinylated anti-goat IgG (1:200) (Jackson ImmunoResearch, West Grove, PA). The monoclonal antibody-treated sections were labeled using the mouse Envision-HRP system (Dako Cytomation, Carpenteria, CA). For all sections, 3,3-diaminobenzidine was used as the indicator substrate, which appeared as a brown reaction product. Slides were counterstained with hematoxylin, mounted, and examined microscopically.

From each treatment group, six cremaster samples were randomly chosen for processing for immunostaining, resulting in a sample size of n=6 per treatment group. Determination of positive staining was performed using the methods of Lefer et al. (Lefer et al., 1998), whereby positive staining was defined as a cremaster microvessel displaying brown reaction product on >50% of the circumference of its endothelium. Cremaster slides were evaluated in a blinded manner. Positive staining is reported as a percentage of the total number of vessels in the cremaster section.

**Statistics**

Data are presented as means±standard error of the mean. For intravital microscopy data, two-group comparisons were performed using Students t-test. Comparison of values in drug-treated
with vehicle treated animals with multiple time points was performed using two-way ANOVA for repeated measures with Bonferroni post-test for comparison of means at each time point. Comparison of parameters over time with baseline within a treatment group was determined using one-way ANOVA using Dunnett’s multiple comparison test. In all cases, a $P$ value ≤0.05 was considered significant.

**Reagents**

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Rosiglitazone maleate was obtained from GlaxoSmithKline.
RESULTS

Physical characteristics, plasma metabolic and blood pressure data.

As shown in Table 2, rat body weight and mean arterial pressure were not different between treatment groups. Plasma free fatty acids were approximately 25% lower in RSG-treated animals compared with vehicle-treated control animals (P<0.01). Fasting plasma glucose, insulin, and adiponectin levels were not affected by RSG treatment.

Venule hemodynamics

Centerline red blood cell velocities and vessel diameters were recorded for each venule to calculate shear rate. Venule diameter (vehicle 45.8±2.5, RSG 44.9±1.0µm), red blood cell velocity (vehicle 2.5±0.1, RSG 2.7±0.1 mm s⁻¹) and shear rate (vehicle 590±35, RSG 650±38 s⁻¹) were not significantly different between vehicle- and RSG-treated groups. The white cell counts (vehicle 9.7±1.1 cellsx10³ /ml, RSG 12.3±2.0 cellsx10³ /ml) were not different between vehicle- and RSG-treated groups.

RSG treatment inhibits ischemia/reperfusion-induced leukocyte-endothelial interactions

Under basal conditions, the rolling leukocyte flux was not different between treatment groups (vehicle 0.5±0.1, RSG 0.6±0.1 cells/WBC). Five minutes following ischemia the rolling leukocyte flux increased 2.2±0.4-fold in vehicle-treated animals and remained elevated throughout the reperfusion period (Fig 1A). In contrast, in RSG treated animals, rolling leukocyte flux did not change appreciably from baseline values throughout the reperfusion period. A significant reduction was observed in the ischemia/reperfusion-induced increase in rolling leukocyte flux at 5 and 90min following reperfusion in RSG-treated animals compared to vehicle-treated controls (Fig 1A). No change in rolling leukocyte velocity was seen over time in either vehicle- or RSG-treated animals (Fig 1B).
In vehicle-treated animals, ischemia/reperfusion induced a large increase in the number of adherent leukocytes, which increased throughout the reperfusion period, reaching a maximum 5-fold increase over preischemia levels at 90 min post-ischemia (Fig 2A). RSG-treatment significantly inhibited this response at 30, 45, 60, and 90 min by 82, 81, 79, and 67%, respectively. While there was apparent suppression in the adhesion response to ischemia at 5 and 15 min, this did not reach statistical significance (Fig 2A). Leukocyte adhesion was reported as number of adherent cells/100\(\mu\)m/WBC rather than fold change because in some cases no adherent cells were observed at time zero, rendering fold change determination inappropriate.

Intravital microscopic images showing the inhibitory effect of RSG on leukocyte adhesion at the 15 and 60 min time point are shown in Fig 2B.

**RSG inhibits ischemia/reperfusion-induced cremaster adhesion molecule expression**

As shown in Fig 3, mRNA expression of VCAM-1 was reduced by 35% \((P<0.01)\) in cremaster tissue from rats treated with RSG compared to vehicle-treated controls. ICAM-1 expression showed a trend suggesting reduced expression in RSG-treated animals. Also in the cremaster, there was no difference in mRNA expression of P-selectin (vehicle 3.73 ±0.05x10^{-3}; RSG 3.27±0.05x10^{-3} mRNA copies/rpl32, \(P=0.25\)) and E-selectin (vehicle 2.01±0.03 x10^{-3}; RSG 1.88±0.03 x10^{-3} mRNA copies/rpl32, \(P=0.38\)) between treatment groups. In aortic tissue, mRNA expression of VCAM-1 and ICAM-1, (Fig 3A and 3B, right panel) were not different between treatment groups. Aortic mRNA expression of P-selectin (vehicle 1.03 ±0.16x10^{-3}; RSG 1.11±0.28x10^{-3} mRNA copies/rpl32, \(P=0.41\)) and E-selectin (vehicle 0.83 ±0.23x10^{-3}; RSG 0.62±0.13x10^{-3} mRNA copies/rpl32, \(P=0.23\)) was also not different between treatment groups.

MCP-1 mRNA expression was measured as a general marker of tissue inflammation. In the cremaster, mRNA expression of MCP-1 was reduced by 25% \((P<0.05)\) in rats treated with...
RSG compared to vehicle controls (Fig 3C). In the aorta, MCP-1 mRNA levels were nearly 100-fold lower than those found in cremaster. RSG treatment was associated with a small but significant increase in MCP-1 mRNA in the aorta (Fig 3C, right panel).

Immunohistochemical staining of frozen sections of cremaster tissue was used to determine the effects of RSG treatment on adhesion molecule protein expression. As shown in Fig 4, both P- and E-selectin immunostaining were significantly reduced by 21% and 61% in RSG-treated samples compared to vehicle (\( P<0.05 \) and \( P<0.001 \), respectively). RSG-treatment was associated with a slight, but not statistically significant reduction in ICAM-1 positive staining compared to vehicle-treated controls (Fig 5A). The amount of vessels positively stained for VCAM-1 was significantly reduced by 50% in RSG-treated samples compared to control (Fig 5B, \( P<0.01 \)).
DISCUSSION

Increased leukocyte adhesion is a marker of an inflamed and dysfunctional endothelium, and is the critical initiation step in atherogenesis (Falk et al., 1995; Berliner et al., 1995). Leukocyte adhesion in response to ischemia/reperfusion is increased in diabetic animals (Granger, 1999), and thus represents a common link between ischemia/reperfusion injury, diabetes, and atherosclerosis. The effects of RSG treatment on leukocyte recruitment have been studied only recently and indirectly. RSG has been shown to reduce expression of adhesion molecules in vitro and in ischemic myocardium in rats (Pasceri et al., 2000; Yue et al., 2001). Histological evidence implies that leukocyte infiltration into atherosclerotic or ischemic tissues is reduced with RSG treatment (Pasceri et al., 2000; Ricote et al., 1998; Yue et al., 2001), but the exact site of action of RSG on the stages of leukocyte recruitment (rolling and/or adhesion) has not been studied to date.

The current study showed that in vehicle-treated animals, immediately following ischemia/reperfusion, leukocyte rolling flux was increased followed by a gradual increase in firmly adherent cells to the endothelium. The selectin family of adhesion molecules is associated with the initial phase of leukocyte recruitment characterized by leukocyte rolling (Ley, 1996). However, rolling leukocyte velocities were not different between treatment groups. While reductions in both P-and E-selectin were seen in the current study, the reduction in E-selectin protein expression with RSG treatment was markedly more profound than that of P-selectin. This is in accordance with the notion that P-selectin is more critical in the initial rolling and slowing of recruited leukocytes (Robinson et al., 1999) while E-selectin is more important in leukocyte arrest, or the transition from slow rolling to firm adhesion, as postulated by Smith et al. (Smith 2004). The inhibitory effect of RSG treatment on firm cell adhesion is consistent with the
reduction in VCAM-1 mRNA and protein expression, as the beta-1 and beta-2 integrin adhesion molecules (VCAM-1, ICAM-1) are critical for firm attachment of activated leukocytes in the recruitment cascade (Ley, 1996). Taken together, these data suggest that the effects of RSG on leukocyte recruitment occur mainly during the transition of leukocyte rolling (E-selectin-mediated) to firm adhesion (VCAM-mediated). While a previous study in normal Lewis rats reported an inhibitory effect of RSG treatment on myocardial ICAM-1 expression (Yue et al., 2001) the current study showed no overt effect on ICAM-1. Potential reasons for a lack of effect on ICAM-1 include differences in study design and rat strain used. It should be noted that while leukocyte-endothelial interactions and leukocyte rolling velocities were monitored throughout the reperfusion period, adhesion molecule mRNA and protein expression were determined following tissue collection at the end of the reperfusion period. The expression data represent a “snap-shot” of the mechanism of RSG protection, however, and a more detailed analysis of the kinetic changes in adhesion molecule expression following reperfusion requires further characterization.

Despite the fact that the stages of leukocyte recruitment (leukocyte rolling, slowing, firm adhesion, extravasation) are similar between venules and arterioles, and that these stages of leukocyte recruitment are critical in atherogenesis (Ross, 1999), extrapolation of the results observed in venules to processes in arterioles or to the atherosclerotic disease state must be performed with appropriate caution, due to differences in vessel wall geometry, blood flow, and regulation of cellular processes between the two vascular beds. Indeed, additional studies are required, using vessels that are more closely relevant to atherosclerosis and preclinical models of atherosclerosis to further characterize the link between the protection observed by RSG in the current study with a potential protective role in atherosclerosis.
The reduction in cremaster MCP-1 expression with RSG treatment is consistent with studies in cultured endothelial cells (Lee et al., 2000; Murao et al., 1999) and in vivo (Yue et al., 2001). MCP-1 has been shown to stimulate recruitment of leukocytes to inflammatory sites (Furie and Randolph, 1995), and could represent a possible mechanism by which RSG inhibits leukocyte recruitment. The physiological relevance of the small but significant increase in aortic MCP-1 expression with RSG treatment is questionable, considering the extremely low expression levels seen in this tissue relative to the cremaster, and considering the body of literature that documents an inhibitory effect of RSG and other thiazolidinediones on MCP-1 expression (Sundararajan and Landreth, 2004). Aortic adhesion molecule mRNA expression was used to determine whether any effects associated with RSG treatment were specific to tissue exposed to ischemia-reperfusion, rather than a global effect on adhesion molecule expression.

Adiponectin has been shown to have insulin-sensitizing and anti-atherogenic properties (Scherer et al., 1995; Hu et al., 1996), and RSG and other PPAR-γ agonists have been shown to stimulate adiponectin expression in vitro (Motoshima et al., 2002; Chinetti et al., 2004). Plasma adiponectin did not change appreciably with RSG treatment, suggesting that upregulation of adiponectin in vivo may require more chronic treatment with RSG, but likely does not contribute to the effects of RSG on leukocyte adhesion in the current study.

In this study, RSG treatment did not affect plasma glucose or insulin levels, which is in agreement with studies that describe anti-diabetic effect of chronic, but not acute treatment of ZDF rats with RSG (Smith et al., 2000). However, a recent study by Yue et al. (Yue et al., 2005) reported a reduction in plasma glucose in 12-14 week old ZDF rats treated with acute RSG treatment. At 16-18 weeks of age, the animals in the current study may have been in a more advanced diabetic state, with pancreatic beta cell destruction and overt insulin resistance, where
further improvement in glucose control was not possible with acute RSG treatment. The reduction in plasma free fatty acids with RSG treatment in the current study might also indicate improvements in insulin sensitivity. Therefore, it is possible that RSG may have had a minor metabolic effect in the current study, with no overt changes in glucose or insulin, though this requires further study.

One limitation of the current study is that the effects of RSG treatment on leukocyte-endothelial interactions and other endpoints were not examined in lean, non-diabetic rats. The present study was not intended to imply that RSG treatment inhibits ischemia-reperfusion-induced leukocyte adhesion solely in diabetic rats. Indeed, it has been shown that RSG protects against myocardial ischemia-reperfusion-induced injury and inflammation in normal rats (Yue et al., 2001). The current study represents an important first step in identifying the in vivo effects of RSG on the inflammatory response to ischemia-reperfusion injury in a model of type II diabetes, and additional studies will be required to more completely characterize the mechanism of the protection in other models of inflammatory vascular disease as well as in normal animals. The data in the current study in ZDF rats suggest that improvements in glucose handling or insulin sensitivity might not be required for protection. However, we believe that the scope of the current study and the findings therein have important clinical implications because leukocyte-endothelial interactions and inflammation are augmented in the diabetic state.

In summary, the current study describes protection by RSG against ischemia-reperfusion-induced leukocyte recruitment in ZDF rats. This study shows, for the first time, the point in the leukocyte recruitment cascade where RSG exerts its protective effect, as well as visualization of the functional benefit of this effect in vivo. Given the critical importance of leukocyte adhesion
in the initiation of atherosclerosis, the main cause of cardiovascular mortality in type 2 diabetes, this study clarifies the mechanism of protection by RSG in cardiovascular disease.
ACKNOWLEDGMENTS

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REFERENCES

*Circulation* 91:2488-2496.


LEGENDS FOR FIGURES

1. RSG treatment inhibits ischemia/reperfusion-induced leukocyte endothelial interactions.

Intravital microscopy of cremaster venules and image analysis was performed as described in Methods. A) Rolling leukocyte flux; baseline (preischemia) values were not different between vehicle- and RSG-treated animals (0.5±0.1 and 0.6±0.1, respectively); B) leukocyte rolling velocity. * P<0.05, ** P<0.01 compared to vehicle-treated group (2-way ANOVA), n=10

2. RSG treatment inhibits ischemia/reperfusion-induced leukocyte adhesion. A) Intravital microscopy of cremaster venules and image analysis was performed as described in Methods. ** P<0.01 compared to vehicle-treated group (2-way ANOVA), n=10. B) Inhibition of ischemia/reperfusion-induced leukocyte adhesion by RSG. Images of cremaster venules taken at 15 min (i and ii), and 60 min (iii and iv) post ischemia, showing adherent leukocytes (arrows) in vehicle treated (i and iii) compared with RSG-treated animals (ii and iv). Scale bar = 20μm.

3. RSG treatment inhibits ischemia/reperfusion-induced adhesion molecule and MCP-1 mRNA expression in ZDF rat cremaster (left panel) and aortic tissues (right panel). mRNA expression for A) VCAM-1, B) ICAM-1, and C) MCP-1 was determined as described in Methods. * P<0.05, **P<0.01 vs vehicle, n=10.

4. RSG treatment inhibits selectin protein expression in cremaster venules. A) and B) P-selectin, C and D) E-selectin. A) and C) Representative photomicrographs of sections
stained for selectins as described in Methods. Scale bar = 50µm. *P<0.05, ***P<0.001 vs vehicle, n=6.

5. RSG treatment inhibits adhesion molecule protein expression in cremaster venules. A) and B) ICAM-1, C and D) VCAM-1. A) and C) Representative photomicrographs of sections stained for ICAM or VCAM, respectively as described in Methods. Scale bar = 50µm.

*P<0.05, n=6.
TABLE 1.
Primer/TaqMan probe sequences (all sequences 5’ to 3’)

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Table 2
Physical characteristics, plasma metabolic and blood pressure data.

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**P<0.01
Figure 1

A

Rolling leukocyte flux

- vehicle
- RSG

B

Leukocyte rolling velocity

- vehicle
- RSG

Figure 1
**Figure 2**

A

**Adherent cell count**

- Vehicle
- RSG

B

**Vehicle**

**RSG**

15min

60min
Figure 3

A  VCAM-1

B  ICAM-1

C  MCP-1

Relative expression (copy number/rpl32)

Vehicle  RSG

Vehicle  RSG

Vehicle  RSG
Figure 4

(A) P-selectin

(B) % positive staining vessels

(C) E-selectin

(D) % positive staining vessels

**Figure 4**
Figure 5

A ICAM-1

Vehicle

RSG

B

% positive staining vessels

Vehicle

RSG

C VCAM-1

Vehicle

RSG

D

% positive staining vessels

Vehicle

RSG

*