Rosiglitazone Protects against Ischemia/Reperfusion-Induced Leukocyte Adhesion in the Zucker Diabetic Fatty Rat


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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. However, the effects of RSG 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 weeks) were treated with RSG (3 mg/kg/day, p.o.) 7 days before experimentation. Leukocyte-endothelial interactions in cremaster venules were recorded using intravital microscopy prior to 30 min of ischemia and during a 90-min reperfusion period. Although 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 vascular cell adhesion molecule-1 (VCAM-1) was reduced by 35% in RSG-treated animals (P < 0.01), whereas P- and E-selectin and intercellular adhesion molecule-1 (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.

Cardiovascular disease is a major cause of mortality in type 2 diabetic patients (Hurst and Lee 2003). Obesity, dyslipidemia, hypertension, and 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 postmyocardial infarction mortality seen in type 2 diabetes (Kereiakes 1985; Woodfield et al., 1996). One of the potential mechanisms that may contribute to vascular disease in 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, 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 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 (Oakes et al., 1994; Fujiwara et al., 1998) and in human patients (Wagstaff and Goa, 2002) are well documented. In addition, thiazolidinediones have been shown to inhibit adhesion molecule expression in vitro (Ricote et al., 1998; Pasceri et al., 2000) 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), although 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 adhesion in 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).

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kocyte 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 Zucker diabetic fatty (ZDF) rat.

Materials and Methods

Animals. All procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals of 1996 and were approved by the GlaxoSmithKline Animal Care and Use Committee. Male ZDF rats (16–18 weeks old) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Rosiglitazone (3 mg/kg/day) or vehicle (0.9% saline, 1% dimethyl sulfoxide v/v) was administered via oral gavage once daily for 7 days prior to experimentation (n = 10 rats/treatment group). This dose was previously shown to protect against myocardial infarction in normal rats (Yue et al., 2001). Rats were fasted overnight before 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., Cleveland, OH). Isoflurane was reduced to 1.5%, and rats were allowed to equilibrate for 10 min. Pressure data were recorded every 10 s for 5 min. The cremaster muscle was prepared as described recently (Johns et al., 2005).

Intravital Microscopy. Intravital microscopy was performed using an upright water immersion microscope (Olympus BX51WI; B&B Microscopes, Warrendale, PA). A 4× objective lens was used to select between three and six 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 QGA; Cooke Corp., Londonderry, NH) coupled to MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). A single bright-field image (20×) was taken of each venule for venule diameter and length measurement. Leukocytes were fluorescently labeled (Rhodamine 6G, 0.3 mg/kg i.v. bolus) before experimentation via a right femoral vein cannula. Labeled leukocytes were epi-illuminated with a mercury arc lamp, 20× water immersion objective lens and rhodamine filter cube. Time-lapse image stacks (1 frame/s for 30 s) and streaming video (66.67 frames/s for 4.5 s) were generated for each venule before 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 to the abdominal cavity. Cessation of blood flow was verified in each vessel visually. In pilot experiments, 30 min of ischemia was found to produce a reproducible increase in leukocyte adhesion without sustained reductions in postreperfusion blood flow as seen in longer ischemia periods. After 30 min of ischemia, clamps were removed and the return of blood flow was verified via microscopic observation. Each venule was recorded at 5, 15, 30, 45, 60, and 90 min postischemia.

After the 90-min 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 time-lapse period (30 s per 100 μm 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 that were moving slower than the centerline blood flow (Gavins and Chatterjee, 2004). For each animal, the rolling velocities of approximately five 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 described previously (Jucker et al., 2002). White blood cell counts were determined with the Advia 120 instrument, according to the manufacturer’s instructions (Buyer Corp.-Diagnostics Div., Tarrytown, NY).

Real-Time Quantitative PCR. Cremaster tissues were homogenized in liquid nitrogen using a mortar and pestle, and total RNA was extracted using RNAzol, according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). Total RNA was extracted from tissues using RNeasy Maxi kit (QIAGEN, Valencia, 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 (Applied Biosystems, 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 reverse transcription-PCR was performed using the ABI Prism 7700 system (Applied Biosystems) as described previously (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 μg/ml), goat polyclonal anti-rat-E-Selectin (15 μg/ml) (all from R&D Systems, Minneapolis, MN), and mouse monoclonal anti-rat ICAM-1 (1 μg/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 (DakoCytomation California Inc., 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 and for immunostaining, resulting in a sample

| Table 1 | Primer/TaqMan probe sequences (all sequences 5′ to 3′) |
|---|---|---|---|
| Gene | Forward | Reverse | TaqMan Probe (5′-FAM, 3′-TAMRA) |
| VCAM-1 | GCCACTTTGACCTCTTGGCTGA | TGTCTAGGAGCTGTCATCCGG | ATCTTCGTCGCTGGCAAGTCCCATCCCATGG |
| ICAM-1 | TGGGTTCTCTCCAAGACGTTC | GCTGGAGACTCTCCAGGCTGT | GTGATACCTCTCTGGACCCCTGTCCT |
| P-selectin | GCCGTATGGCAGAGACAGTCC | TAAGCGGAGCAGCTGTCGTC | TGTGTTAAGGTCCTCCAGACATGTTGCCC |
| E-selectin | CCGGATGCTGCTTCTGCTGTGA | TAAGGTGTCGCTCCAGAGA | TGTGAGTGCTTAACTCTG |
| MCP-1 | CCAAATCTGAGATGCTGTCGCA | CACAGTGCAAGGTCGGAAGTCC | TGGACGAGAAAATGAGATCAGAAAA |

FAM, carboxyfluorescein; TAMRA, carboxytetramethylrhodamine.
size of n = 6 per treatment group. Determination of positive staining was performed using the methods of 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 ± S.E.M. For intravital microscopy data, two-group comparisons were performed using Student’s 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’s 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 and 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 ± 1.0 μm), 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 cells x 10³/ml; RSG 12.3 ± 2.0 cells x 10³/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/white blood count). Five minutes after 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 90 min after reperfusion in RSG-treated animals compared with 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 postischemia (Fig. 2A). RSG treatment significantly inhibited this response at 30, 45, 60, and 90 min by 82, 81, 79, and 67%, respectively. Although 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 a number of adherent cells/100 μm² white blood count, rather than -fold change, because in some cases, no adherent cells were observed at time 0, 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 <

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**Fig. 1.** RSG treatment inhibits ischemia/reperfusion-induced leukocyte endothelial interactions. Intravital microscopy of cremaster venules and image analysis were performed as described under Materials and 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 with vehicle-treated group (two-way ANOVA), n = 10.

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TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight</th>
<th>Mean Arterial Pressure</th>
<th>Insulin</th>
<th>Glucose</th>
<th>Adiponectin</th>
<th>Free Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>mm Hg</td>
<td>ng/ml</td>
<td>ng/dl</td>
<td>µg/ml</td>
<td>mM</td>
</tr>
<tr>
<td>Vehicle</td>
<td>366 ± 19</td>
<td>118 ± 6</td>
<td>1.4 ± 0.3</td>
<td>729 ± 25</td>
<td>5.0 ± 0.4</td>
<td>1.2 ± 0.10</td>
</tr>
<tr>
<td>RSG</td>
<td>345 ± 12</td>
<td>129 ± 3</td>
<td>1.2 ± 0.2</td>
<td>704 ± 23</td>
<td>6.0 ± 0.5</td>
<td>0.9 ± 0.03**</td>
</tr>
</tbody>
</table>

**P < 0.01.**
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, immunostaining of both P- and E-selectin was significantly reduced by 21 and 61% in RSG-treated samples compared with 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 with vehicle-treated controls (Fig. 5A). The amount of vessels positively stained for VCAM-1 was significantly reduced by 50% in RSG-treated samples compared with 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 (Berliner et al., 1995; Falk 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 (Ricote et al., 1998; Pasceri et al., 2000; 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 after 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. Although 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), whereas E-selectin is more important in leukocyte arrest or the transition from slow rolling to firm adhesion, as postulated by Smith et al. (2004).

The inhibitory effect of RSG treatment on firm cell adhesion is consistent with the reduction in VCAM-1 mRNA and protein expression, because the β-1 and β-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). Although 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, although leukocyte-endothelial interactions and leukocyte rolling velocities were monitored throughout the reperfusion period, adhesion molecule mRNA and protein expression were determined after tissue collection at the end of the reperfusion period. However, the expression data represent a "snapshot" of the mechanism of RSG protection, and a more detailed analysis of the kinetic changes in adhesion molecule expression after reperfusion requires further characterization.

Despite the fact that the stages of leukocyte recruitment (leukocyte rolling, slowing, firm adhesion, and 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 because of 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 and a potential protective role in atherosclerosis.

The reduction in cremaster MCP-1 expression with RSG treatment is consistent with studies in cultured endothelial cells (Murao et al., 1999; Lee et al., 2000) 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
Rosiglitazone Inhibits Leukocyte Adhesion in ZDF Rats in Vivo

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 antiatherogenic properties (Scherer et al., 1995; Hu et al., 1996), and RSG and other peroxisome proliferator-acti-
vated receptor-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 up-regulation of adiponectin in vivo may require more chronic treatment with RSG but probably 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 antidiabetic effect of chronic but not acute treatment of ZDF rats with RSG (Smith et al., 2000). However, a recent study by Yue et al. (2005) reported a reduction in plasma glucose in 12- to 14-week-old ZDF rats treated with acute RSG treatment. At 16 to 18 weeks of age, the animals in the current study may have been in a more advanced diabetic state, with pancreatic β 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 overall changes in glucose or insulin, although 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 nondiabetic 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.

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References


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