Rosuvastatin Treatment Reverses Impaired Coronary Artery Vasodilation in Fructose-fed, Insulin Resistant Rats

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Running Head: Rosuvastatin Improves Vasodilation in Insulin Resistance.

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Abstract.

Insulin resistance (IR) impairs vascular responses in coronary arteries, but mechanisms of dysfunction and approaches to treatment remain unclear. We examined the ability of a new HMG-CoA reductase inhibitor, rosuvastatin, to reverse reduced dilator responses in rats made IR by feeding a fructose-rich diet (FF). Sprague Dawley rats were randomized to control (normal rat diet) or FF. After one week, rats received rosuvastatin (2mg/kg) or placebo (saline) subcutaneously for 5 weeks. Biochemical measurements and in vitro functional studies of small coronary arteries were performed. Fasting insulin and triglyceride (TG) levels were markedly increased in FF-placebo rats as compared to other groups. Rosuvastatin treatment of FF rats normalized TG and modestly decreased insulin levels. Acetylcholine (ACh) induced dilator responses were depressed in arteries from FF-placebo rats. This impairment was due to decreased responses via calcium-dependent K channels (K\textsubscript{Ca}). Rosuvastatin treatment of FF rats completely reversed the response to ACh to normal levels. Moreover, this recovery in function was due to an improvement in vasodilation via K\textsubscript{Ca}. Thus, rosuvastatin treatment of IR rats normalizes coronary vascular dilator responses by improving the K\textsubscript{Ca} function.

Keywords: Fructose-fed rat, insulin resistance, coronary arteries, rosuvastatin, calcium-dependent potassium channels
Introduction.

Epidemiologic and experimental studies provide strong evidence linking insulin resistance to the development of cardiovascular disease. Several prospective studies, in which fasting insulin concentrations were used as an index of insulin sensitivity, found that elevated insulin levels are associated with increased hypertension, ischemic heart disease, and cardiovascular death (26, 13, 7). Moreover, a direct correlation has been described between insulin resistance and carotid intima media thickness (18). Thus, these data provide strong evidence that insulin resistance is an important risk factor for the development of cardiovascular disease.

Previous studies have demonstrated that vascular responses to endothelium-dependent vasodilators are markedly impaired in small coronary, cerebral, and mesenteric arteries from fructose-fed, insulin resistant rats (28, 21, 12). Moreover, it has been shown that this vascular derangement is primarily due to a defect in the vascular smooth muscle $K_{Ca}$ channels (28-29, 8). To date, there is no known specific treatment for this vascular dysfunction. The 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are a class of lipid lowering agents that are known to lower cardiovascular morbidity and mortality in diabetic patients (16, 15). Although the mechanism of this effect is closely related to their ability to lower lipids, there are also thought to be positive non-lipid effects of these agents (4, 22). One specific property is their ability to improve endothelial function. This effect appears to be due to their ability to up-regulate nitric oxide synthase (NOS) expression and to decrease free radical production (4, 22, 24, 17, 5). Obviously, these pharmacologic mechanisms may have positive effects on vascular
dysfunction in insulin resistance. Thus, the purpose of this study is to assess the effect of chronic rosuvastatin treatment on lipids, insulin, and coronary vascular function in fructose-fed, insulin resistant rats.

Methods.

The protocol was approved by the Institutional Animal Care and Use Committee of Wake Forest University School of Medicine. All experiments complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Male Sprague-Dawley rats were obtained at 6 weeks of age and randomized into one of the following two groups: 1) normal diet (control; n = 24) or 2) fructose-fed (FF; n = 24) rats. After one week of the respective diets, rats were further randomized to receive either rosuvastatin (2 mg/kg) or placebo (volume matched saline) subcutaneously for a period of 5 weeks. This dose was based on recommendations from AstraZeneca, who have performed complete dose ranging studies during research and development of this agent. The route of administration was chosen based upon ease to the investigators; however, we were assured by AstraZeneca, based on their previous studies, that this route maintained comparable bioavailability to oral administration. The animals in the fructose-fed group were fed a fructose-rich diet containing 66% fructose, 22% casein, and 12% lard, plus essential vitamins and minerals (Teklad Labs; Madison, WI), whereas control animals received standard rat chow. Previous studies in our laboratory and others have shown that receiving the fructose-rich diet induces a state of insulin resistance, which is characterized by glucose intolerance, hyperinsulinemia, and hypertriglyceridemia (28,
19). We have shown that hyperinsulinemia occurs within 7 days of beginning the diet (20).

After completion of the treatment protocol, fasted rats were anesthetized with pentobarbital (50 mg/kg IP) and anticoagulated with heparin (500 units IP). A midline incision was made and a 1 ml blood sample was withdrawn from the heart for biochemical analysis. Subsequently, the heart was removed and placed in a chilled oxygenated modified Krebs-Ringers bicarbonate solution (millimolar concentration: NaCl, 118.3; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; KH2PO4, 1.2; NaHCO3, 25; dextrose, 11.1). Small coronary arteries (≈150 µm diameter) from the septum and/or the left ventricular free wall were isolated from surrounding perivascular tissue and removed. Intraluminal diameter of pressurized (60mmHg) arteries were measured as previously described (28, 21, 12). Only one concentration-response experiment was performed per arterial segment, however; several arterial segments were taken from each rat.

**Protocol.** Coronary arteries were allowed to equilibrate for 30 minutes in the tissue bath. Subsequently, vessels were preconstricted to 30 - 50 % of their resting diameter with endothelin-1 (≈10^{-8} M). Concentration-response studies to acetylcholine (10^{-9} to 3x10^{-5} M), an endothelium-dependent vasodilator were performed. To evaluate the role of the specific endothelium-derived relaxing factors several inhibitors were used. To evaluate nitric oxide in the vascular response to acetylcholine, arteries were pretreated for a period of 30 minutes with N-nitro-L-arginine (LNNA) (100 µM), an inhibitor of nitric oxide synthase. To evaluate the role of prostacyclin, indomethacin (100 µM), an inhibitor of cyclooxygenase, was added to the bath for 30 minutes prior to the determination of acetylcholine induced relaxations. Finally, to evaluate the role of
endothelium-derived hyperpolarization via calcium-activated potassium channels (\(K_{Ca}\)) in the vascular response to acetylcholine, arteries were pretreated with charybdotoxin (CTX) (0.1 \(\mu\)M) and apamin (0.5 \(\mu\)M) for a period of 30 minutes prior to acetylcholine dose-response experiments. The combination of these two inhibitors were chosen based on evidence showing that they consistently inhibit \(K_{Ca}\)-mediated relaxation and based on our prior experiments with these agents (28, 12, 2, 9).

**Biochemical Measurements.** Plasma insulin was assayed by using a dextran-coated charcoal immunoassay with rat antibody. Glucose concentrations were measured using a Glucose Trinder Kit (Sigma Chemicals, St. Louis, MO). Lipid concentrations (including triglycerides and total cholesterol) were measured using a Dimension® Clinical Chemistry System (Newark, DE) with specific reagent kits for each lipid determination.

**Chemicals.** All chemicals used in this study were obtained from Sigma Chemicals (St. Louis, MO). All agents were dissolved in deionized water and diluted with Krebs buffer. N-nitro-L-arginine was dissolved in water and titrated to a pH of approximately 2 with hydrochloric acid for dissolution. The pH was then titrated to physiologic level (7.4) with sodium hydroxide.

**Data Analysis.** Statistical analysis of the concentration response experiments were performed using a two-factor ANOVA with repeated measures. Statistical comparisons for baseline diameter and biochemical measurements were performed using a two-factor ANOVA followed by a Tukey’s test for pairwise comparisons. Data are reported as mean ± SEM. The criteria for significance was \(p<0.05\).
Results.

Biochemical Results

Fasting insulin, glucose, total cholesterol, triglycerides, and body weights for all experimental groups are shown in table 1. Fasting glucose, total cholesterol, and body weight were not significantly different in any of the groups. Both fasting insulin and triglycerides were significantly increased in the FF-placebo group. Treatment of the FF rats with rosuvastatin reversed the triglyceride levels to normal levels, while the insulin concentrations, although significantly reduced, remained markedly elevated.

Vascular Reactivity Experiments. Resting intraluminal diameter of small coronary arteries did not differ between groups (180±7 μm for control-placebo, 192±8μm for control-rosuvastatin, 180±5μm for FF-placebo and 190±5 μm for FF-rosuvastatin arteries, P=NS). It should be noted that the application of the pharmacologic inhibitors did not significantly alter the resting diameter as compared to the arteries without pharmacologic intervention. Moreover, percent arterial constriction after endothelin was similar among groups with 44±2 % for control-placebo, 41±2 % for control-rosuvastatin, 44±3 % for FF-placebo and 43±2 % for FF-rosuvastatin arteries (P=NS). Furthermore, it should be noted that the amount of ET-1 used for preconstriction did not differ among the groups.

Acetylcholine induced a concentration-dependent vasodilation in all groups (figure 1); however, vasodilation to acetylcholine in the FF-placebo group was markedly reduced in comparison to the remaining groups. It should be noted that following treatment with rosuvastatin, vasodilation to acetylcholine in coronary arteries from FF
rats was normalized. Studies with LNNA demonstrated that acetylcholine-induced vasodilation in both the control-placebo and control-rosuvastatin groups was significantly impaired by the inhibition of NOS (figure 2). In contrast, inhibition of NOS in the FF-placebo group almost completely abolished acetylcholine-induced relaxation, where the maximal relaxation was $54 \pm 7\%$ for acetylcholine alone and $11 \pm 8\%$ in the presence of LNNA (figure 2). Again, treatment of FF animals with rosuvastatin reversed the acetylcholine response (in the presence of LNNA) back to normal levels (maximal relaxation = $67 \pm 7$; figure 2). Pretreatment of arteries with CTX + apamin combination treatment decreased the relaxation to acetylcholine in both the control-placebo and control-rosuvastatin groups and the FF-rosuvastatin group to a similar degree (figure 3 compared to figure 1). In contrast, CTX + apamin pretreatment of the arteries from the FF-placebo group had little effect on acetylcholine induced relaxation (maximal relaxation: $55 \pm 9\%$ in the presence of CTX/apamin and $54 \pm 7\%$ for acetylcholine alone, NS) (figure 3 compared to figure 1). Pretreatment with indomethacin had no effect on acetylcholine induced relaxation in either the control or FF groups (data not shown).
Discussion

The purpose of the present study was to evaluate the effectiveness of chronic treatment with rosuvastatin on coronary vascular dysfunction, lipids, and insulin concentrations in the fructose-fed rat model of insulin resistance. We have shown that after 5 weeks of treatment with rosuvastatin, triglyceride concentrations were normalized in the FF, insulin resistant rats. Moreover, hyperinsulinemia, a marker for insulin resistance, was modestly improved after rosuvastatin therapy, while normal total cholesterol levels were unchanged. Regarding vascular reactivity, we have shown that vasodilation to acetylcholine was normalized in coronary arteries from rosuvastatin treated, FF rats. Furthermore, the current data suggest that rosuvastatin treatment improves vascular responsiveness through its ability to restore $K_{Ca}$ channel function to normal levels.

Rosuvastatin is a new HMG-CoA reductase inhibitor. This class of agents, commonly referred to as “statins”, have been reported to have both direct and indirect effects on vascular function (24). Indirectly, statins have been shown to improve vascular function and cardiovascular outcomes through their ability to improve lipid profiles, including the reduction of triglycerides (14, 25). In addition, there is a plethora of literature suggesting that the statins have many direct vascular effects. These activities include increased upregulation or activation of endothelial NOS, reduced oxidative stress, and decreased inflammatory responses (4, 22, 24, 17, 5). It is unclear from the current data if the normalization of vascular function observed is due to direct vascular effects of rosuvastatin or indirect effects due to the fact that it normalized triglyceride concentrations.
Hypertriglyceridemia is a commonly occurring dyslipidemia that often accompanies insulin resistance and type 2 diabetes mellitus and is an obvious metabolic derangement in the current model. Previous studies have shown that in patients with isolated hypertriglyceridemia or in hypertriglyceridemia associated with type 2 diabetes mellitus, lowering triglycerides improves endothelium dependent vasodilation (3, 6). The mechanism of this improvement in vascular function is purportedly due to a decrease in oxidative stress, though other mechanisms may be operating (23). Therefore, the reduction of triglycerides seen in the current study may be directly related to the improvement in vascular function.

Using pharmacologic tools, we have previously demonstrated in small coronary, mesenteric, and cerebral arteries from fructose-fed, insulin resistant rats that vasodilation via $K_{Ca}$ channels is nearly abolished (28, 21, 7). Moreover, using patch clamp techniques, we have shown that in vascular smooth muscle cells from mesenteric arteries of fructose-fed rats, large conductance $K_{Ca}$ ($BK_{Ca}$) channel activity is markedly reduced (8). In the current study, treatment with rosuvastatin was able to normalize acetylcholine mediated vasodilation via the $K_{Ca}$ channel. Since the underlying mechanism of $K_{Ca}$ channel dysfunction in this model is unknown, it is difficult to speculate on the mechanism of rosuvastatin’s effect, however, based on data from others, it may be through the reduction of oxidative stress. Previous data have demonstrated that vascular dysfunction of aorta from fructose-fed, insulin resistant rats results from increased free radical production (31, 30). Moreover, it has been shown that the functional activity of the $BK_{Ca}$ channel is suppressed by increased oxidative stress (27, 32). Thus, it is possible, though not studied, that rosuvastatin improves vascular function by reducing
oxidative stress since this is a known direct effect of statin agents and of normalizing triglycerides.

There have been two previous studies to assess the role of statin treatment on vascular function in insulin resistant animal models (fructose-fed rat and spontaneously hypertensive rat (SHR)) (10, 1). Both studies demonstrated that endothelium-dependent vasodilation is improved in large conduit arteries (carotid and aorta, respectively) after statin therapy. However, these studies differ from our own in that their data suggests that endothelium-dependent vasodilation was restored through increased production of NO. This mechanism is not surprising since several statins have been shown to directly increase NO production/activity and since conduit arteries are primarily dependent on NO, as opposed to $K_{Ca}$ channels, for endothelium-dependent vasodilation. The current data differs from the above studies in the fact that it demonstrates the normalization of triglyceride levels with rosuvastatin therapy (neither of the previous studies measured this factor) and that the mechanism of this effect is directed toward improved function of $K_{Ca}$ channels. This difference in mechanism may be a reflection on the size or type of artery studied.

In conclusion, rosuvastatin treatment of fructose-fed, insulin resistant rats completely reverses both hypertriglyceridemia and coronary vascular dysfunction. Moreover, it appears that rosuvastatin may normalize coronary vascular responsiveness through its effects on $K_{Ca}$ channel function. These are the first data to demonstrate this effect by a statin and may prove to be an important clinical feature of rosuvastatin in the future.
References


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Figure Legends

**Figure 1.** Cumulative concentration-response experiments to acetylcholine in small coronary arteries from control and FF rats treated with placebo or rosuvastatin. (*) indicates statistically significant reduction (p<0.05) in vasodilation compared to the remaining groups.

**Figure 2.** Cumulative concentration-response experiments to acetylcholine in LNNA pretreated small coronary arteries from control and FF rats treated with placebo or rosuvastatin. (*) indicates statistically significant reduction (p<0.05) in vasodilation compared to the remaining groups.

**Figure 3.** Cumulative concentration-response experiments to acetylcholine in charybdotoxin/apamin (CTX+AP) pretreated small coronary arteries from control and FF rats treated with placebo or rosuvastatin. (*) indicates statistically significant reduction (p<0.05) in vasodilation compared to the remaining groups.
Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight (gms)</th>
<th>Glucose (mg/dl)</th>
<th>Insulin (pg/ml)</th>
<th>Total Cholesterol (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control – Saline</td>
<td>359 ± 6</td>
<td>101 ± 3</td>
<td>771 ± 140</td>
<td>66 ± 6</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Control – Rosuvastatin</td>
<td>358 ± 6</td>
<td>93 ± 3</td>
<td>810 ± 95</td>
<td>64 ± 2</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>FF – Saline</td>
<td>362 ± 4</td>
<td>107 ± 7</td>
<td>2279 ± 153*</td>
<td>63 ± 4</td>
<td>106 ± 13*‡</td>
</tr>
<tr>
<td>FF - Rosuvastatin</td>
<td>351 ± 6</td>
<td>93 ± 5</td>
<td>1889 ± 194*†</td>
<td>67 ± 3</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

- * indicates p<0.01 versus control-saline group
- † indicates p<0.01 versus FF-saline group.
- ‡ indicates p <0.01 versus FF-rosuvastatin group.
Figure 1
Figure 2

Acetylcholine (log M)

% Relaxation

- Control - Placebo (n=6)
- Control - Rosuvastatin (n=7)
- FF - Placebo (n=6)
- FF - Rosuvastatin (n=9)

**All arteries pretreated with LNNA prior to acetylcholine.

* p < 0.05
** p < 0.01
Figure 3.

**All arteries pretreated with CTX + AP prior to acetylcholine.