

Effects of Rosuvastatin on Nitric Oxide–Dependent Function in Aorta and Corpus Cavernosum of Diabetic Mice

Relationship to Cholesterol Biosynthesis Pathway Inhibition and Lipid Lowering

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Elevated plasma lipids contribute to neurovascular dysfunction in diabetes. Statins have lipid-lowering properties and can modulate endothelial nitric oxide (NO) bioavailability. The aim was to assess the impact of these factors on autonomic nitrergic nerve and endothelial function. Thus, the effects of diabetes and treatment with the HMG-CoA reductase inhibitor rosuvastatin (RSV) were examined on corpus cavernosum and aorta from streptozotocin-induced diabetic mice in a 4-week prevention study and a 2-week intervention study, following 4 weeks of untreated diabetes. Cotreatment with mevalonate was used to assess the dependence of RSV's effects on HMG-CoA reductase blockade. Diabetes caused a 25% reduction in NO-mediated endothelium-dependent relaxation to acetylcholine for aorta and cavernosum. Relaxations of cavernosum were in the nondiabetic range following prevention or reversal treatment. The aortic deficit was completely prevented and 60% reversed by RSV. Maximum NO-dependent nonadrenergic, noncholinergic nerve-mediated relaxations of cavernosum were reduced 25–33% by diabetes. RSV treatment prevented 75% and reversed 71% of this diabetic deficit. Cotreatment with mevalonate inhibited the beneficial actions of RSV on aorta and cavernosum. Total plasma cholesterol was unaltered by diabetes or treatment. Thus, RSV corrected defective NO-mediated nerve and vascular function in diabetic mice independent of cholesterol lowering but via effects dependent on cholesterol biosynthesis pathway inhibition. *Diabetes* 52:2396–2402, 2003

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ACh, acetylcholine; EDR, endothelium-dependent relaxation; EFS, electrical field stimulation; NANC, nonadrenergic and noncholinergic; RSV, rosuvastatin; SNP, sodium nitroprusside; STZ, streptozotocin.

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Diabetic patients have an increased risk of vascular and nerve dysfunction. Vasculopathy results in part from endothelial cell abnormalities involving reduced production or action of vasodilators, such as nitric oxide (NO), and altered responses to vasoconstrictors (1,2). Hyperglycemia, oxidative stress, and altered lipid profiles contribute to vascular complications, including peripheral nerve perfusion deficits, which play an important role in the etiology of diabetic neuropathy (2–4). Indeed, epidemiological studies have identified dyslipidemia as an independent, potentially modifiable, risk factor for diabetic neuropathy (2,5).

Aortas from several diabetic animal models show decreased NO-mediated endothelium-dependent relaxation (EDR) to agonists such as acetylcholine (ACh) (6–10); similar deficits have been reported in diabetic patients (2,11,12). In addition, both endothelial and nonadrenergic and noncholinergic (NANC) nerve-derived NO-mediated smooth muscle relaxation is diminished by diabetes in corpus cavernosum of animal models and humans (13–18). Normal erectile function involves nerve-mediated increases in arterial inflow to corpus cavernosum, relaxation of smooth muscle, and restriction of venous outflow. NANC nerves provide the majority of NO during erection; however, neuropeptides and vasodilators released from the endothelium (including NO) also have a physiological role (13,14).

The most common drugs used in hyperlipidemic patients, including those with diabetes, are HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase inhibitors (statins), which inhibit the rate-limiting step in cholesterol biosynthesis. Statins reduce LDL and VLDL levels, while modestly increasing HDL (19,20). This protects against coronary artery disease in diabetes (20–23). In a small, randomized, double-blind study, rosuvastatin (RSV) decreased total plasma LDL cholesterol and triglycerides in hyperlipidemic type 2 diabetic patients (24).

Experimentally, statins modulate endothelial function, including increases in NO production, at doses insufficient to lower plasma lipids (25–28). However, the detailed effects of statins on vascular function in diabetes remain to be fully elucidated. Moreover, it is not known whether

TABLE 1
Body weights and plasma glucose concentrations of mice in all groups

Group	<i>n</i>	Body weight (g)		Plasma glucose (mmol/l)
		Start	End	
Nondiabetic mice				
Control	31	35.8 ± 0.9	—	12.6 ± 0.4
RSV treatment	14	35.8 ± 1.3	35.7 ± 1.1	12.9 ± 0.8
Diabetic mice				
4 weeks	16	33.8 ± 1.4	26.9 ± 1.1*	42.3 ± 1.8†
6 weeks	12	38.2 ± 2.2	29.0 ± 1.3*	42.9 ± 2.7†
Prevention RSV treatment	14	32.4 ± 1.1	28.2 ± 1.2*	39.7 ± 2.4†
Prevention RSV + mevalonate cotreatment	12	35.8 ± 1.3	29.9 ± 1.0*	35.9 ± 1.4†
Intervention RSV treatment	12	41.0 ± 2.2	30.4 ± 1.0*	38.3 ± 1.8†

Data are means ± SE. **P* < 0.001 vs. start weight; †*P* < 0.001 vs. nondiabetic control group.

statins influence the neuronal NO system in parallel to an action on endothelium, which could have important implications for diabetic impotence. Therefore, the aim was to assess the effects of diabetes and RSV treatment on mouse corpus cavernosum and aorta function.

RESEARCH DESIGN AND METHODS

Male C57BL/6J mice from the University of Aberdeen breeding colony were aged 4–6 months on the day of experimentation. They had ad libitum access to standard laboratory diet and water. Experiments were performed in accordance with regulations specified by the U.K. Animal Procedures Act (1986) and the National Institutes of Health Principles of Laboratory Animal Care (1985 revised version).

Unless otherwise stated, all chemicals were obtained from Sigma (Poole, Dorset, U.K.). Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (AstraZeneca, Macclesfield, Cheshire, U.K.) dissolved in sterile saline at a dose of 125 mg/kg. After 48 h, diabetes was verified by the presence of hyperglycemia and glycosuria (Visidex II and Diastix; Ames, Slough, U.K.) in nonfasted mice. Diabetic mice were weighed daily and were rejected if body weight increased over 3 consecutive days or if blood glucose was <20 mmol/l.

Diabetes duration was 4 or 6 weeks, and both prevention and intervention studies were undertaken. The prevention study comprised a group of diabetic control mice untreated for 4 weeks; a second diabetic group received RSV (Crestor; AstraZeneca) treatment as an oral gavage (20 mg · kg⁻¹ · day⁻¹ in 0.5% Tween solution) after 48 h, following confirmation of STZ-induced diabetes. This dose was chosen based on the results of a dose-response study where it gave ~95% correction of a sciatic nerve motor conduction velocity deficit in rats and on efficacy for inhibition of hepatic cholesterol biosynthesis in rodents (29,30). A further 4-week diabetic group received RSV (20 mg · kg⁻¹ · day⁻¹) and cotreatment with mevalonate (150 mg · kg⁻¹ · day⁻¹ in the drinking water). In addition, a group of nondiabetic mice received RSV treatment (20 mg · kg⁻¹ · day⁻¹) for 4 weeks. The intervention study used 6-week untreated diabetic controls and a second diabetic group that was given RSV (20 mg · kg⁻¹ · day⁻¹) for 2 weeks following 4 weeks of untreated diabetes.

On the day of experiments mice were anesthetized (5% halothane in air, with 0.1 ml 10% urethane in saline per 10 g body wt i.p.). The thoracic aorta was removed between the aortic arch and the diaphragm. The penis was excised at its base with removal of the glans penis and connective and adventitial tissues along the shaft. Blood was taken from the heart, and plasma was frozen for subsequent plasma glucose, total cholesterol, and total triglyceride determination by means of colorimetric spectrophotometry using standard test kits (Sigma, Poole, Dorset, U.K.).

Aorta experiments. The thoracic aorta was cleared of connective tissue and two sections cut into 1-mm lengths were mounted as ring preparations, using 40-μm tungsten wire, into 5-ml organ baths in a small-vessel myograph for measurement of isometric tension. Aortas were bathed in carboxygenated (95% O₂:5% CO₂) modified Krebs-Ringer solution (144 NaCl, 5 KCl, 1.25 Ca²⁺, 1.1 MgSO₄, 25 NaHCO₃, 1.1 NaH₂PO₄ and 5.5 glucose: all in mmol/l) at 37°C (pH 7.35). Resting tension was maintained at 0.75 g. Tissues were left to equilibrate for 1 h, with frequent changing of bathing solution, before tissue viability was assessed for contractile responses and endothelial integrity, with 300 nmol/l phenylephrine followed by 100 nmol/l ACh. After a 45- to 60-min washout and recovery period, cumulative concentration response curves to phenylephrine were determined. This was followed by precontraction with phenylephrine at ~80% of the maximal response to give a background tension

against which to assess concentration response relationships for ACh and then sodium nitroprusside (SNP). In some experiments, concentration response curves were repeated after a 30-min incubation period with 10 μmol/l of the cyclooxygenase inhibitor flurbiprofen or the NO synthase inhibitor NG-nitro-L-arginine (L-NNA).

Corpus cavernosum experiments. Individual corpus cavernosum were separated and mounted in 10-ml organ baths and bathed in Krebs-Ringer solution as for aorta. Tension was monitored by isometric transducers, and resting tension was set at 0.5 g. Tissues were left to equilibrate for 1 h before determination of cumulative concentration response curves for phenylephrine and for SNP against an ~80% maximum phenylephrine precontraction. Tissues were washed and allowed to recover for 20–30 min between response curves. Noncumulative concentration response curves for ACh were established against individual 80% maximal phenylephrine precontractions.

Repetitive supramaximum transmural electrical field stimulation (EFS) of autonomic nerves was delivered via platinum wire electrodes placed on either side of the cavernosum (train duration 30 s, frequency 2–30 Hz, and pulse duration 5 ms, 90 mA). Frequency response curves were determined for noradrenergic vasoconstrictor nerves before incubation with atropine (1 μmol/l) and guanethidine (4 μmol/l) for 30 min to eliminate responses mediated by cholinergic and noradrenergic nerves, respectively. Following phenylephrine precontraction, EFS produced frequency-dependent relaxations mediated by NANC nerves. Some concentration or frequency response curves were repeated following a 30-min incubation period with 10 μmol/l flurbiprofen or L-NNA. At the end of the experiments tissues were lightly patted dry and weighed.

Statistical analysis. Data are expressed as means ± SE. They were subjected to Bartlett's test for homogeneity of variances before ANOVA. Where significance was reached (*P* < 0.05), between-group differences were established using the Newman-Keuls multiple comparison test. Otherwise, data were analyzed by Kruskal-Wallis nonparametric ANOVA and Dunn's multiple comparison test. Concentration response data were fitted by sigmoid curves using the least squares method to calculate EC₅₀. Whole-curve analyses comparisons were made using two-way ANOVA. For comparisons between two groups of data only, paired or unpaired two-tailed Student's *t* tests were used. All calculations were made using a standard software package (Prism3; Graphpad, San Diego, CA).

RESULTS

Diabetic groups developed a more than threefold increase in plasma glucose concentrations (*P* < 0.001) and had 25–35% (*P* < 0.001) weight loss (Table 1); neither parameter was significantly altered by RSV or RSV with mevalonate cotreatment. Total plasma cholesterol was 5.0 ± 0.3 mmol/l (*n* = 18) in the control group. This was unaffected by 4-week diabetes (4.7 ± 0.4 mmol/l, *n* = 16) or RSV treatment (nondiabetic 4.5 ± 0.4 mmol/l, *n* = 14; diabetic 4.6 ± 0.5 mmol/l, *n* = 14). Triglyceride levels were unaffected by diabetes (control 1.13 ± 0.19 mmol/l, *n* = 13; 4-week diabetes 1.12 ± 0.24 mmol/l, *n* = 12), but were ~40% reduced by RSV treatment (nondiabetic 0.64 ± 0.07 mmol/l, *n* = 12; 4-week diabetes 0.61 ± 0.21 mmol/l, *n* =

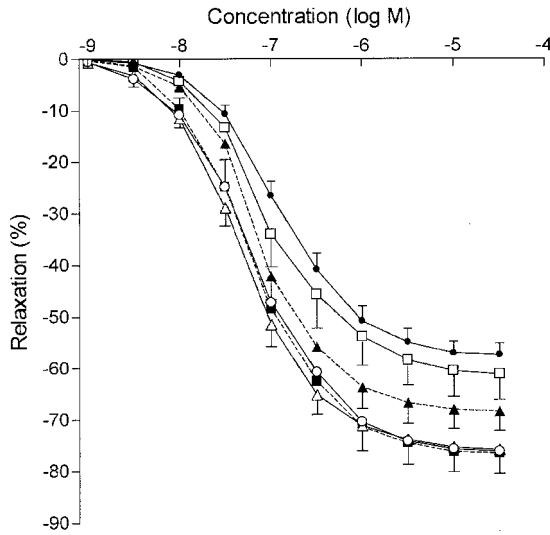


FIG. 1. Cumulative concentration response curves for relaxation to acetylcholine for aortas from nondiabetic and diabetic mice and the effects of prevention RSV treatment without and with mevalonate cotreatment and intervention RSV treatment. ○, nondiabetic control, *n* = 16; △, nondiabetic group treated with 20 mg · kg⁻¹ · day⁻¹ RSV for 4 weeks, *n* = 11; ●, data from 4- (*n* = 15) and 6-week (*n* = 10) diabetic control groups did not differ significantly and have been pooled for clarity; -■-, prevention diabetic group treated with 20 mg · kg⁻¹ · day⁻¹ RSV for 4 weeks from induction, *n* = 13; □, diabetic group cotreated with 20 mg · kg⁻¹ · day⁻¹ RSV and 150 mg · kg⁻¹ · day⁻¹ mevalonate from induction, *n* = 11; -▲-, intervention diabetic group treated with 20 mg · kg⁻¹ · day⁻¹ RSV for 2 weeks following 4 weeks of untreated diabetes, *n* = 11. Data are mean ± SE.

7), although this only reached statistical significance (*P* < 0.05) in the treated nondiabetic group.

Aorta study. Maximum EDR to ACh (Fig. 1), following phenylephrine precontraction, was similarly and significantly reduced by 4 and 6 weeks of diabetes compared with nondiabetic controls (56.9 ± 3.4, *n* = 15, and 58.1 ± 2.9, *n* = 10, vs. 76.2 ± 3.4%, *n* = 16; *P* < 0.001). RSV treatment completely prevented the diabetic deficit (76.5 ± 3.9%, *n* = 13, *P* < 0.01) (Fig. 1A). Mevalonate cotreatment at ~79% attenuated (61.1 ± 5.1%, *n* = 11, *P* < 0.05) the effect of RSV alone. Maximum relaxation for RSV-treated nondiabetic rats (75.9 ± 3.8%, *n* = 11) did not differ from that of untreated controls. In the intervention group, RSV gave ~57% reversal (68.4 ± 3.8%, *n* = 11) of the diabetic deficit to the extent that the relaxation did not differ significantly from the nondiabetic control value. Incubation with 10 μmol/l flurbiprofen did not alter ACh-mediated responses of control (*n* = 11) or 4-week diabetic (*n* = 9) mice (data not shown). In contrast, addition of L-NNA completely abolished ACh-mediated relaxations of control and 4-week diabetic aortas (data not shown). Diabetes tended to slightly reduce sensitivity to ACh, assessed by (-log)EC₅₀, although this did not reach statistical significance. Thus, all group (-log)EC₅₀ values were in the range of 7.32 ± 0.10 for the RSV-treated control group and 6.87 ± 0.13 for 4-week diabetic group.

Maximum endothelium-independent relaxation (~95–99% for all groups) and sensitivity ([-log]EC₅₀, ~7.9–8.0 for all groups) to the NO donor SNP following phenylephrine precontraction were unaltered by diabetes or by treatment (Table 2). However, in the presence of 10 μmol/l L-NNA, while there was no effect on maximum relaxation, there were small but significant increases in sensitivity to

TABLE 2
Effects of diabetes, RSV, and 10 μmol/l L-NNA administration on aorta relaxation responses to SNP and contractile responses to phenylephrine

Group	SNP			Phenylephrine		
	n	Maximum relaxation (%)	(-log) EC ₅₀	n	Maximum contraction (mN)	(-log) EC ₅₀
Nondiabetic mice						
Control	14	95.6 ± 1.1	7.91 ± 0.08	13	8.75 ± 0.96	6.86 ± 0.10
RSV treatment	10	98.6 ± 0.6	7.92 ± 0.07	9	9.01 ± 1.04	6.93 ± 0.14
Diabetic mice						
4 weeks	10	95.2 ± 1.8	7.87 ± 0.08	12	13.42 ± 0.95	7.62 ± 0.10§
6 weeks	9	95.4 ± 1.4	8.03 ± 0.09	9	14.25 ± 1.04	7.63 ± 0.07§
Prevention RSV treatment	12	98.3 ± 0.5	7.99 ± 0.08	13	12.14 ± 0.73#	7.33 ± 0.14#
Prevention RSV + mevalonate cotreatment	9	98.4 ± 0.8	7.86 ± 0.06	10	11.91 ± 0.83#	7.23 ± 0.13
Intervention RSV treatment	11	95.5 ± 1.2	7.90 ± 0.06	10	12.60 ± 0.74#	7.47 ± 0.15
				+ L-NNA	+ L-NNA	+ L-NNA
				8.18 ± 0.08†	11.20 ± 1.49†	7.06 ± 0.10‡
				8.11 ± 0.09†	10.96 ± 1.12*	6.95 ± 0.13
				7.91 ± 0.08	14.03 ± 0.98	7.88 ± 0.08†
				8.07 ± 0.10	15.54 ± 1.19	7.62 ± 0.10
				8.11 ± 0.08‡	13.61 ± 0.86†	7.44 ± 0.17
				8.05 ± 0.08†	13.51 ± 1.11‡	7.29 ± 0.13
				8.05 ± 0.09‡	15.10 ± 1.27‡	7.57 ± 0.16

Data are means ± SE. **P* < 0.001, †*P* < 0.01, ‡*P* < 0.05 vs. response prior to L-NNA administration; §*P* < 0.001, ||*P* < 0.01, #*P* < 0.05 vs. the nondiabetic control group.

SNP in nondiabetic controls ($P < 0.01$), RSV-treated nondiabetic controls ($P < 0.01$), and prevention ($P < 0.05$) and intervention ($P < 0.05$) in RSV-treated diabetic groups. However, this effect was absent in aorta from 4- or 6-week diabetic mice. Cotreatment with mevalonate did not alter the L-NNA-mediated response ($P < 0.01$) for prevention in RSV-treated diabetic mice.

Maximum contraction to phenylephrine increased by >1.5 -fold ($P < 0.01$) by 4 and 6 weeks of diabetes compared with nondiabetic controls (Table 2). Neither preventive RSV without or with mevalonate cotreatment nor intervention RSV treatment significantly altered the effects of diabetes on maximum contraction. RSV treatment of nondiabetic mice was also without effect. Similarly, sensitivity to phenylephrine, as measured by ($-\log$) EC_{50} values, increased by ~ 0.7 log units after 4 and 6 weeks of diabetes ($P < 0.001$), and this was not significantly altered by treatment.

After preincubation with $10 \mu\text{mol/l}$ L-NNA, maximum contractions to phenylephrine of aorta (Table 2) from nondiabetic mice were ~ 1.3 -fold ($P < 0.01$) increased. In contrast, there was no significant effect of L-NNA on 4- and 6-week diabetic responses. RSV prevention ($P < 0.01$) and intervention ($P < 0.05$) treatments partially restored the augmented contractions in the presence of L-NNA. Cotreatment with mevalonate did not alter the augmented phenylephrine response ($P < 0.05$) of prevention RSV-treated diabetic mice. Similar to nontreated controls, RSV-treated nondiabetic aortas developed a ~ 1.2 -fold ($P < 0.001$) increase in maximum contraction with L-NNA. Both nondiabetic controls ($P < 0.05$) and 4-week diabetic ($P < 0.01$) but not 6-week diabetic aortas developed an increased sensitivity to phenylephrine in the presence of L-NNA. Similar trends were observed for the other groups, though statistical significance was not attained.

Corpus cavernosum study. Tissue weights did not differ significantly between groups (7.0 – 7.5 mg, data not shown). Transmural EFS of corpus cavernosum caused frequency-dependent contractions that were abolished by incubation with $4 \mu\text{mol/l}$ guanethidine. Expressed relative to tissue weight, maximum contractions at 30 Hz were not significantly different between groups: tensions in mN/mg were in the range 0.059 ± 0.006 (prevention RSV-treated diabetic group, $n = 13$) to 0.078 ± 0.005 (RSV-treated nondiabetic group, $n = 12$).

EFS following phenylephrine precontraction in the presence of $1 \mu\text{mol/l}$ atropine and $4 \mu\text{mol/l}$ guanethidine produced frequency-dependent NANC relaxations (Fig. 2) that could be completely abolished by L-NNA (data not shown). Relaxations for diabetes of 4 and 6 weeks duration did not differ significantly and resulted in 25 and 33% reductions in maximum (20 Hz) relaxation compared with the nondiabetic control group ($46.4 \pm 3.4\%$, $P < 0.01$, and $40.8 \pm 3.9\%$, $P < 0.001$, vs. $61.1 \pm 3.3\%$), RSV treatment prevented 75% ($57.3 \pm 3.3\%$, $P < 0.05$) and reversed 71% ($55.2 \pm 3.6\%$, $P < 0.05$) of these diabetic deficits. Maximum NANC relaxation of RSV-treated nondiabetic mice ($63.6 \pm 1.7\%$) did not differ from that of the control group. Mevalonate cotreatment of RSV-treated diabetic mice gave a maximum relaxation value ($45.6 \pm 3.7\%$) that was not significantly different from that of the 4-week diabetic

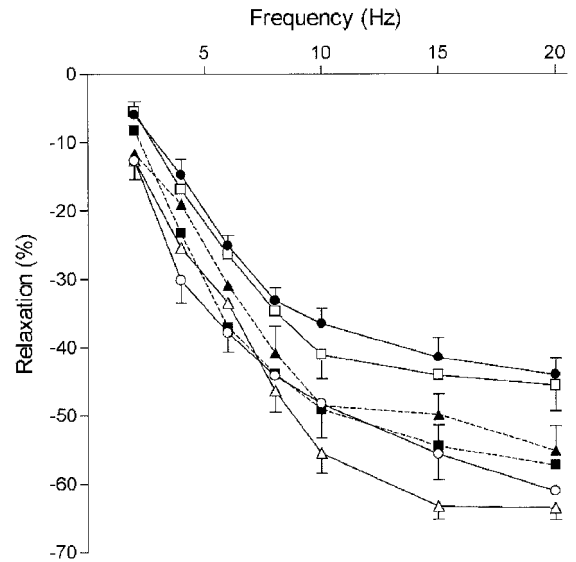


FIG. 2. Frequency response curves for relaxation to transmural electrical field stimulation for corpus cavernosum from nondiabetic and diabetic mice in the presence of atropine and guanethidine and the effects of prevention RSV treatment without and with mevalonate cotreatment and intervention RSV treatment. \circ , nondiabetic control, $n = 16$; \triangle , nondiabetic treated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV for 4 weeks, $n = 12$; \bullet , data from 4- ($n = 12$) and 6-week ($n = 9$) diabetic control groups did not differ significantly and have been pooled for clarity; \blacksquare , prevention diabetic group treated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV for 4 weeks from induction, $n = 14$; \square , diabetic cotreated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV and $150 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ mevalonate from induction, $n = 12$; \blacktriangle , intervention diabetic group treated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV for 2 weeks following 4 weeks with untreated diabetes, $n = 9$. Data are means \pm SE.

group but was reduced compared with RSV treatment alone ($P < 0.05$).

After phenylephrine precontraction, EDR in response to ACh (Fig. 3) after 4 and 6 weeks of diabetes revealed similar significant whole-curve deficits ($P < 0.001$) compared with controls. While RSV did not alter responses from nondiabetic tissues, treatment largely prevented ($P < 0.001$) the diabetic deficit, although relaxation across the concentration range remained slightly depressed compared with the nondiabetic control curve ($P < 0.05$). Cotreatment with mevalonate blocked the action of RSV, such that responses did not differ from those of diabetic controls. In addition, RSV partially reversed the diabetic curve deficit ($P < 0.05$), although responses remained lower than those of nondiabetic controls ($P < 0.01$).

Maximum endothelium-independent relaxation and sensitivity to SNP following phenylephrine precontraction of corpus cavernosum were unaltered by diabetes, RSV treatment, and RSV and mevalonate cotreatment. Maximum relaxations did not differ significantly between groups and were in the range $47.0 \pm 4.0\%$ (intervention RSV) to $55.1 \pm 3.8\%$ (nondiabetic control). Similarly, ($-\log$) EC_{50} values ranged from 6.08 ± 0.15 (intervention RSV) to 6.24 ± 0.09 (RSV-treated nondiabetic). In addition, L-NNA preincubation failed to alter SNP-mediated relaxations in any group (data not shown).

The maximum corpus cavernosum contractile responses and ($-\log$) EC_{50} values for phenylephrine were not altered by diabetes or treatment. Thus, maximum tensions for all groups ranged from 0.122 ± 0.007 (RSV-treated nondiabetic group) to 0.096 ± 0.007 mN/mg (pre-

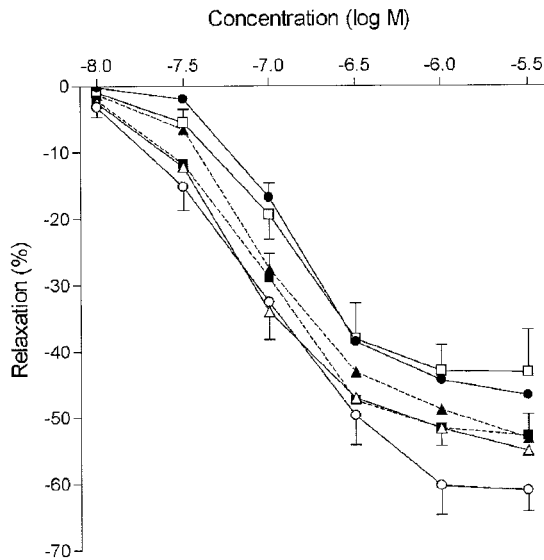


FIG. 3. Concentration response curves for relaxation to acetylcholine for corpus cavernosum from nondiabetic and diabetic mice and the effects of prevention RSV treatment without and with mevalonate cotreatment and reversal RSV treatment. \circ , nondiabetic control, $n = 14$; \triangle , nondiabetic group treated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV for 4 weeks, $n = 13$; \bullet , data from 4- ($n = 12$) and 6-week ($n = 9$) diabetic control groups did not differ significantly and have been pooled for clarity; \blacksquare , prevention diabetic group treated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV for 4 weeks from induction, $n = 11$; \square , diabetic group cotreated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV and $150 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ mevalonate from induction, $n = 10$; \blacktriangle , intervention diabetic group treated with $20 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ RSV for 2 weeks following 4 weeks of untreated diabetes, $n = 10$. Data are means \pm SE.

ventive RSV-treated diabetes). Similarly, $(-\log) \text{EC}_{50}$ values ranged from 6.07 ± 0.05 (nondiabetic control) to 6.27 ± 0.06 (preventive RSV-treated diabetes). L-NNA preincubation did not significantly alter corpus cavernosum maximum tension or $(-\log) \text{EC}_{50}$ to phenylephrine.

DISCUSSION

Diabetes caused defective NO-mediated EDR to Ach stimulation in mouse aorta. This is the first report for STZ-induced diabetic mice and is in agreement with data from type 2 diabetic C57/BL/KsJ-*db/db* mice (8) and diabetic rats (2,6,7). Corpus cavernosum EDR was also impaired, in agreement with one report for diabetic mice (16) and several for rat, rabbit, and humans (15,17,18,31). EDR of mouse aorta and corpus cavernosum was unaffected by cyclo-oxygenase inhibition but was completely abolished by NO synthase blockade, as noted for rat aorta and corpus cavernosum (7,17). This suggests that in mouse aorta and corpus cavernosum, EDR is mediated by NO rather than prostanoids. Endothelium-independent relaxation to the NO donor SNP was unaffected by diabetes in both tissues, indicating that the smooth muscle cGMP mechanism was intact, in agreement with findings from diabetic nonmurine species (6–8,15–18). In diabetic patients, some studies have reported impaired endothelium-independent responses to NO donors (11,32,33), while other have not (12,18). The reason for this discrepancy is unclear but may reflect the severity and progression of vascular damage.

RSV treatment prevented and largely reversed the corpus cavernosum and aorta EDR deficits. This is the first report of statin effects on these parameters in experimen-

tal diabetes. Previous work showed that statins alter expression of several genes controlling vascular tone, inflammation, and coagulation in cultured human umbilical vein endothelial cells under normoglycemic conditions. Thus, mRNA for NO synthase-3 was increased, while endothelin-1 mRNA levels were reduced. In addition, mRNAs for interleukin-8, monocyte chemoattractant protein-1, and plasminogen activator inhibitor-1 were elevated, whereas thrombomodulin mRNA was reduced (34). Furthermore, statins protected cultured bovine aortic endothelial cells against adverse changes in NO synthase and endothelin-1 expression when challenged with oxidized LDL (35). In humans, statins reduce levels of circulating C-reactive peptide, an effect independent of lipid lowering (36), and decrease levels of soluble cell adhesion molecules (37). There are also complex statin effects on adhesion molecule expression by cultured endothelial cells (38). Thus, the prediction is that statins may protect vascular endothelium function in diabetes. However, a recent report failed to find any effect of atorvastatin on reduced forearm blood flow responses to endothelium-dependent and endothelium-independent agonists in type 2 diabetic patients (32). The reason for the discrepancy with the mouse data is unclear, but it may be that any statin effect on endothelial NO production in those patients was obscured by the blunted responses to NO donors also noted in that study, which does not occur in mice.

In patients with type 2 diabetes, plasma VLDL levels are typically higher, those of HDL are lower, and those of total and LDL cholesterol are about the same as in the nondiabetic population (20). However, LDL particles are smaller, denser, and more glycated and oxidized in diabetes, making them a risk factor for vascular disease. Together, LDL and VLDL triglycerides impair endothelium-dependent vasodilation by altering NO production (9,10,39,40). Under basal conditions, endothelial NO synthase-3 is targeted to cholesterol-rich membrane domains characterized by the presence of caveolin, which competes with calmodulin for NO synthase binding, perhaps explaining the inverse relationship between circulating cholesterol levels and NO activity (39,41).

In hypercholesterolemic patients, forearm blood flow responses to Ach infusion and NOS inhibition were reduced, suggesting decreased NO production (40). In addition, impaired EDR was found in vessels from hyperlipidemic-diabetic hamsters and rabbits (9,10) and hypertriglyceridemic rats (42). Although there may be indications of reduced NO production, enhanced inactivation of NO by reactive oxygen species is probably the most important mechanism by which NO-dependent endothelial function is blunted by diabetes (2). Indeed, antioxidants improve aorta and corpus cavernosum function of diabetic animals (2,4,7,9,16,17).

Total plasma cholesterol and triglyceride levels were unaltered by diabetes in mice, in agreement with results for several mouse strains (43). RSV had no effect on cholesterol levels, which are considerably lower in mice than humans, although there was a tendency toward lower triglycerides. While STZ-induced diabetic mice are not hyperlipidemic, they may be dyslipidemic; increased LDL and decreased HDL cholesterol has been reported in

STZ-induced diabetic mice (44), though diabetes duration was twice that in the present study. In contrast to mice, 8-week STZ-induced diabetic rats have a 70% increase in total cholesterol and a sevenfold elevation of VLDL triglycerides (29). Very-high-dose RSV partially corrected the triglyceride elevation but was without effect on cholesterol. In these rats, treatment with RSV dose-dependently corrected nerve conduction velocity deficits with marked effects at doses that did not alter lipid levels. Thus, in both diabetic mice and rats, statin effects on neurovascular function can occur independently of their lipid-lowering action.

Increased endothelial NO production may account for the observed improvements in EDR, as RSV has been shown to elevate NO synthase-3 expression in mouse aorta without altering total plasma cholesterol and triglycerides (26–28). Statins also attenuate rolling, adherence, and transmigration of leukocytes (45), inhibit superoxide production, and reduce LDL oxidation (46,47) independent of lipid lowering.

The beneficial effects of RSV were markedly attenuated by cotreatment with mevalonate, the product of HMG-CoA reductase. Thus, mevalonate inhibited RSV action on aortic and cavernosal EDR and cavernosal NANC-mediated relaxation. This suggests that RSV effects are dependent upon cholesterol biosynthesis pathway inhibition, but independent of cholesterol lowering. Statins inhibit the synthesis of several metabolites downstream of mevalonate, including farnesyl and geranylgeranyl pyrophosphate. These cholesterol synthesis intermediates can isoprenylate proteins and have important roles in endothelial function (25–28). For example, RhoGTPases undergo geranylgeranyl modification, which is necessary for their membrane-associated activity. They negatively regulate NO synthase-3 expression in cultured human endothelial cells. Thus, by blocking Rho geranylgeranylation, statins can upregulate endothelial NO synthase-3 (48). However, investigations on such pleiotropic actions of statins have not been carried out in diabetic models or even under hyperglycemic conditions in tissue culture, and any change in the isoprenylation state of important regulatory proteins is unknown.

A notable finding was that RSV largely prevented and reversed the impaired NO-mediated NANC relaxation of corpus cavernosum smooth muscle. Statin effects on NANC nerves have not previously been reported. The data suggest that statins modulate the activation or expression of NO synthase in nitrergic nerves in diabetes. While the liver is the major target for cholesterol lowering by statins, HMG-CoA reductase is expressed and cholesterol is synthesized by most cells, including neural tissue (49). Thus, it is plausible that the mechanisms responsible for statin-induced improvements of nitrergic nerve function in diabetes are direct and mechanistically similar to those for endothelial NO synthase-3 activation, involving altered isoprenylation and upregulation of NO synthase-1 activity. However, there are no published data on direct measurement of neuronal NO synthase-1 activity following statin therapy in the absence of diabetes, and an indirect vascular mechanism may also plausibly explain the present findings. As the nerve vascular supply is important for the development of diabetic neuropathy (2–4,29), the possibil-

ity that RSV improved endothelial function to an extent sufficient to protect major pelvic ganglion perfusion cannot be discounted—autonomic ganglion blood flow is halved by diabetes in rats (50). Such an effect would indirectly protect NANC nerve function. RSV vascular effects in brain are sufficient to protect against experimental ischemic stroke damage in nondiabetic mice (27).

RSV did not significantly alter the diabetes-induced increase in aorta responses to phenylephrine. This suggests that the mechanism responsible was not dependent on NO-linked dysfunction and remains to be established. Responses to exogenous phenylephrine or nerve-released norepinephrine were unaltered by diabetes or L-NNA in mouse corpus cavernosum, implying that the mechanism resulting in increased phenylephrine responsiveness in aorta is not present in penile tissue. However, L-NNA augmented the phenylephrine response of nondiabetic but not diabetic aorta, suggesting that basal NO release was diminished by diabetes. This is further supported by the increased sensitivity to SNP of nondiabetic but not diabetic aorta, observed following L-NNA. RSV promoted control-like responses to L-NNA, suggesting that the effect depended on improved NO bioavailability. There were no alterations of phenylephrine or SNP responses by L-NNA for corpus cavernosum. This suggests that under the in vitro experimental conditions, basal NO release is minimal in corpus cavernosum compared with aorta.

In summary, prevention and intervention RSV treatment improved aorta and corpus cavernosum NO-dependent function in diabetic mice; these benefits were largely attenuated by mevalonate cotreatment. RSV did not alter total plasma cholesterol in this model. Together the data suggest that statin effects on endothelial and nitrergic nerve dysfunction are independent of cholesterol lowering but act through inhibition of the cholesterol biosynthesis pathway. RSV may be suitable for further studies on diabetic neurovascular dysfunction, including clinical trials. The pleiotropic mechanisms of statin action suggest that the development of selective inhibitors of isoprenoid biosynthesis within the mevalonate-cholesterol pathway may potentially lead to further novel therapeutic approaches to diabetic vascular and nerve complications.

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