Resveratrol Inhibits Neointimal Growth after Arterial Injury in High-Fat-Fed Rodents: The Roles of SIRT1 and AMPK

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Keywords

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Abstract

We have shown that both insulin and resveratrol (RSV) decrease neointimal hyperplasia in chow-fed rodents via mechanisms that are in part overlapping and involve the activation of endothelial nitric oxide synthase (eNOS). However, this vasculoprotective effect of insulin is abolished in high-fat-fed insulin-resistant rats. Since RSV, in addition to increasing insulin sensitivity, can activate eNOS via pathways that are independent of insulin signaling, such as the activation of sirtuin 1 (SIRT1) and AMP-activated kinase (AMPK), we

speculated that unlike insulin, the vasculoprotective effect of RSV would be retained in high-fat-fed rats. We found that high-fat feeding decreased insulin sensitivity and increased neointimal area and that RSV improved insulin sensitivity ($p < 0.05$) and decreased neointimal area in high-fat-fed rats ($p < 0.05$). We investigated the role of SIRT1 in the effect of RSV using two genetic mouse models. We found that RSV decreased neointimal area in high-fat-fed wild-type mice ($p < 0.05$), an effect that was retained in mice with catalytically inactive SIRT1 ($p < 0.05$) and in heterozygous SIRT1-null mice. In contrast, the effect of RSV was abolished in AMPK α 2-null mice. Thus, RSV decreased neointimal hyperplasia after arterial injury in both high-fat-fed rats and mice, an effect likely not mediated by SIRT1 but by AMPK α 2.

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Introduction

Metabolic syndrome is defined by a cluster of abnormalities including alterations in glucose tolerance, dyslipidemia, hypertension, and central obesity [1]. Insulin resistance, which is the key characteristic of metabolic syndrome and type 2 diabetes, increases the risk for both atherosclerosis and restenosis after percutaneous revascularization interventions [2, 3].

Insulin has both growth-promoting and protective effects in the vasculature. For instance, high-dose insulin promotes vascular smooth muscle cell (VSMC) proliferation, but increases VSMC differentiation *in vitro* [4, 5]. *In vivo*, we have shown that high-dose systemic insulin (5 U/day) decreases neointimal growth after arterial injury (a model of restenosis) despite increasing VSMC proliferation [6, 7], consistent with a prevalent vasculoprotective effect [6–11]. We have also shown that this vasculoprotective effect is mediated by endothelial nitric oxide synthase (eNOS) [11] but is greatly diminished in insulin-resistant high-fat-fed rats [6] that have low eNOS activation [12]. Therefore, alternative mechanisms to up-regulate NO production independent of insulin or to decrease neointimal growth by pathways unrelated to insulin may be beneficial in insulin-resistant models.

Resveratrol (RSV, trans-3,5,4'-trihydroxystilbene), a polyphenol found in red wine, has cardiovascular protective effects [13], including an “anti-restenotic” effect demonstrated by us as well as others [14–22]. In chow-fed (insulin sensitive) rodents, we have shown that the effect of RSV to decrease the arterial-injury-induced neointimal hyperplasia involves the activation of eNOS [14] by mechanisms that are unclear and potentially independent of insulin. Furthermore, RSV prevents fat-induced insulin resistance in insulin target tissues [23, 24]. Therefore, we hypothesized that the effect of RSV to decrease neointimal hyperplasia may be preserved in a rat model of high-fat diet-induced insulin resistance, where the insulin-induced activation of eNOS is impaired.

RSV can directly or indirectly activate the NAD⁺-dependent class III histone deacetylase sirtuin 1 (SIRT1) [25–27]. SIRT1 has been shown to deacetylate and activate eNOS [28], and whole body or smooth muscle cell-specific overexpression of SIRT1 protected mice against neointimal hyperplasia after arterial injury [29, 30].

SIRT1 can activate liver kinase B1 (LKB1) by deacetylation and thus activate AMP-activated kinase (AMPK) [31]. RSV can also activate AMPK independent of SIRT1 by multiple mechanisms [31–34], including an increase in AMP:ATP ratio due to mitochondrial electron trans-

port chain complex III inhibition [34] and activation of CaMKII [33]. In turn, AMPK can phosphorylate and activate eNOS [35]. Endothelial-specific overexpression of constitutively active AMPK α 1 decreased neointimal hyperplasia in mice [36]. AMPK α 2-null mice had increased neointimal area and intimal cell proliferation after arterial injury [37]. Therefore, the objectives of the present study were: (1) to examine whether RSV decreases neointimal hyperplasia after carotid artery balloon injury in rodents fed a high-fat diet, a model where the “anti-restenotic” action of insulin is impaired, and (2) to determine the role of SIRT1 and AMPK in the action of RSV to decrease neointimal hyperplasia in this model.

To determine the role of SIRT1, we examined the effect of RSV using two genetically modified mouse models. The first model is the catalytically inactive SIRT1 mouse model. This mouse carries a point mutation (H355Y) in the SIRT1 catalytic site, which does not affect SIRT1 protein expression [38]. Since some of the effects of SIRT1 may not be due to its deacetylase activity [39, 40], we also used a heterozygous SIRT1-null mouse model (SIRT1^{+/-}) carrying an allele with a truncated SIRT1 protein [41]. The heterozygous SIRT1-null mice were used because the homozygous knockout mice die postnatally due to developmental defects [41].

To determine the role of AMPK, we chose the AMPK α 2-null mouse, because although supraphysiological AMPK α 1 activation decreased neointimal hyperplasia [36], whole-body AMPK α 2 knockout but not AMPK α 1 knockout was previously found to exacerbate neointimal formation [37].

Materials and Methods

Studies were conducted according to protocols approved by the Animal Care Committee of the University of Toronto. All surgical procedures were done under general anesthesia using isoflurane. Analgesic (Buprenorphine) was given at the onset of each surgical procedure. Male animals were studied as they were the models previously used by our group to investigate the effect of insulin [6–11] and RSV [14] since estrogen affects neointimal growth [42].

Rat Studies

Four groups of Sprague-Dawley rats (Charles River) initially weighing 400–450 g were subjected to the following treatments: (1) vehicle control treatment and a normal-fat control diet (NFC; Dyets, Bethlehem, PA, USA, Dyets No: 100741, 12% calories from fat as corn oil); (2) vehicle control treatment and a high-fat diet (HFC; Dyets No. 100742, same protein and micronutrient composition as the normal-fat diet, 60% calories from fat obtained from beef tallow in addition to the corn oil); (3) RSV treatment (4 mg/kg per day *s.c.*, a dose we previously found to be effective at decreasing

neointimal area in chow-fed rats [14]; Sigma) and a normal-fat diet (NFR); and (4) RSV treatment and a high-fat diet (HFR). RSV dissolved in polyethylene glycol-300 and DMSO (9:1) or the respective vehicle was delivered subcutaneously via an osmotic pump (Alzet; Durect Corp., Cupertino, CA, USA). The implantation of osmotic pumps was performed two days after the diet onset and three days before carotid artery balloon injury, as described previously [14].

Rat Carotid Artery Balloon Injury

Injury to the left common carotid artery was performed as previously described [8].

Hyperinsulinemic-Euglycemic Clamp

To assess the effect of RSV on insulin sensitivity, hyperinsulinemic-euglycemic clamp was performed in pre-cannulated rats that did not receive arterial injury. This is the gold standard measure of insulin sensitivity and is based on the principle that in order to counteract the glucose-lowering effect of a fixed dose of insulin, a variable glucose infusion is required, higher when the subject is more insulin sensitive. Vessel cannulation (carotid artery for blood sampling and jugular vein for insulin/glucose infusion) was performed three days before the clamp procedure, as described previously [7]. After an overnight fast, a fixed rate of insulin (Humulin R, 5 mU/kg per min) was initiated through the jugular vein and maintained for 2 h. Blood samples were taken every 5 min for measuring plasma glucose levels on an Analox rapid Analyzer in order to determine the rate of glucose to be infused to maintain basal glycemia. As indicated above, the rate of glucose infusion represents insulin sensitivity.

Blood Pressure Analysis

The systolic blood pressure was measured indirectly via the tail-cuff technique (Visitech BP-2000 Blood Pressure Analysis System, Apex, NC, USA). Blood pressure was measured before treatment and at 4, 14, and 28 days after arterial injury.

Mouse Studies

To address the role of SIRT1 in RSV's effect, we used two genetic mouse models on different genetic backgrounds: (1) the catalytically inactive SIRT1 homozygous mice (SIRT1^{Y/Y} on Sv129xCD1 background) [38] and their wild-type littermate controls and (2) the heterozygous SIRT1-null mice (SIRT1^{+/-} on Sv129 background) [41] and their wild-type littermate controls. As mentioned in the Introduction, heterozygous mice were used since the homozygous knockout mice die postnatally due to developmental defects [41]. A portion of the SIRT1^{Y/Y} also dies postnatally but most do survive to adulthood [38]. To address the role of AMPK in the effect of RSV, we used whole-body AMPK α 2-null mice (on C57BL/6 background) and wild-type controls. All three genetically modified mouse models display exacerbated insulin resistance on a high-fat diet [43–45]. The mice were fed a high-fat diet (Dyets No. 101920, 23% protein, 14% carbohydrate, and 60% fat mainly from hydrogenated coconut oil) or a 0.0375% RSV-supplemented high-fat diet (Dyets No. 182110) starting from 6 weeks of age and were maintained on their respective diets for the rest of the treatment period. Similar diets [16] have been previously used in mice where similar RSV doses (~36 mg/kg per day) have been found effective to decrease neointimal growth by us and other authors [14, 16].

Mouse Femoral Wire Injury

Mice underwent wire injury of the femoral artery at 7 weeks of age and 5 days after the onset of diets. Wire injury was performed as described previously [14] with the following modifications: (1) In the SIRT1^{Y/Y} and the AMPK α 2-null and their controls, reinsertion of the wire was performed two more times for 1 min each. This was performed to ensure complete removal of the endothelium as mice were bigger than those used in the previous study [14]. (2) In the heterozygous SIRT1-null and littermate controls, one passage of the wire with 1 min wait time, as in the previous study, was enough to denude the artery [14].

Blood and Vessel Sample Collection

Blood glucose was monitored throughout the treatment using a glucometer (OneTouch; LifeScan Inc.) and measured at the same time of the day (~10:00 a.m.). These values were converted to plasma glucose assuming a normal hematocrit. At 28 days after arterial injury, rats or mice were anesthetized with a cocktail of ketamine:xylazine:acepromazine (87:1.7:0.4 mg/mL, 1 μ L/g body weight) to obtain blood samples via cardiac puncture for metabolic parameter analysis. Thereafter, the animals were euthanized by anesthetic overdose. The blood vessels were perfusion-fixed for 4 min (rats) or 15 min (mice) with 10% neutral buffered formalin at physiological pressure (110–120 mm Hg). The vessels were removed, immersed in formalin for 24–48 h, embedded in paraffin, and divided into two blocks by cutting the midsection of the artery. Cross sections were taken from the midsection.

Histomorphometric Analysis

Intimal and medial cross-sectional areas were measured on hematoxylin and eosin-stained slides (rats) or elastic Van Gieson-stained slides (mice) and analyzed using a computer-assisted morphometric system (NIS-Elements BR 3.0; Nikon Corporation). Intimal area was measured as the area encompassed by the internal elastic lamina minus the lumen area and was calculated as the average of two separate cross sections 12 μ m apart. Medial area was measured as the area encompassed by the external elastic lamina subtracted by the area enclosed by the internal elastic lamina and was not different among treatments.

Immunohistochemistry

Phosphorylated eNOS (p-eNOS) and phosphorylated vasodilator-stimulated phosphoprotein (p-VASP) were determined using anti-p-eNOS antibody (1:50; Santacruz, CA, USA) and p-VASP antibody (1:50; Abcam, ON, Canada), as described previously [46]. Immunostaining-positive area was quantified with Image J software (U.S. National Institutes of Health, MD, USA). p-eNOS-positive area was limited to the luminal surface, whereas p-VASP-positive area was observed both in the neointima and media. Therefore, changes in p-eNOS and p-VASP were evaluated as p-eNOS-positive area/lumen length and p-VASP-positive area/whole vascular wall area, respectively.

Western Blot Analysis

Frozen aorta collected at the end of the hyperinsulinemic clamp in rats was homogenized and processed as in our previous studies [11, 12, 14]. Protein concentration was measured by the Bradford assay using bovine serum albumin as a standard. Protein samples (50 μ g) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred

to a nitrocellulose membrane, and analyzed with anti-SIRT1 (1:500), anti-phospho (Thr172) AMPK (1:500), anti-total AMPK (1:1,000), anti-phospho (Ser79) acetyl-CoA carboxylase (ACC) (1:1,000), anti-total ACC (1:1,000), anti-phospho eNOS (1:500), anti-total eNOS (1:500), anti-phospho (Ser473) Akt (1:1,000), anti-total Akt (1:1,000), anti-total I κ B α (1:1,000), and anti-tubulin (1:5,000). All primary antibodies were purchased from Cell Signaling Technology. Secondary antibodies were from Santa Cruz Biotechnology.

Plasma Assays

Since animals were not fasted before sacrifice, metabolic parameters were obtained in the random fed state, which is the physiological state under which the injured vessels of rodents develop neointimal hyperplasia. Fasting insulin and glucose were measured in rats undergoing the hyperinsulinemic clamp. Plasma insulin was determined by radioimmunoassay (Linco Research Inc., St. Charles, MO, USA). Plasma free fatty acids (Wako, Osaka, Japan) and plasma triglycerides (Roche Diagnostic, Indianapolis, IN,

Fig. 1. The effect of resveratrol (RSV) on plasma glucose, plasma insulin, and whole-body insulin sensitivity in rats treated with normal- or high-fat diet. Plasma glucose (a) and insulin (b) levels throughout the 2 h hyperinsulinemic-euglycemic clamp. Whole-body insulin sensitivity is indicated by the glucose infusion rate (GINF) during the 2 h hyperinsulinemic-euglycemic clamp (c). NFC, normal-fat control; HFC, high-fat control; NFR, normal-fat with RSV; HFR, high-fat with RSV. Numbers in parentheses represent rat number. *** $p < 0.001$ HFC versus NFC, $^{\S}p < 0.05$ HFC versus HFR (post hoc Bonferroni's test after a significant interaction was found between diet and RSV, analysis performed on the last 30 min).

Fig. 2. The effect of resveratrol (RSV) on neointimal area in rats fed a normal- or high-fat diet. Representative images ($\times 40$) (a) and quantification of neointimal area (b) of carotid artery cross sections measured 28 days after balloon injury from normal-fat- and high-fat-fed rats treated with RSV or vehicle. Arrows point to in-

ternal elastic lamina. NFC, normal-fat control; HFC, high-fat control; NFR, normal-fat with RSV; HFR, high-fat with RSV. Numbers in parentheses represent rat number. *** $p < 0.001$ HFC versus NFC, $^{\S\S}p < 0.01$ HFC versus HFR (post hoc Bonferroni's test after a significant interaction was found between diet and RSV).

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a

b

USA) were measured using colorimetric kits. Plasma NO was determined as total nitrates + nitrites via a colorimetric assay kit (QuantiChrom Nitric Oxide Assay kit; BioAssay Systems, MA, USA).

Statistical Analysis

Two-way ANOVA (diet and RSV status for rats, and genotype and RSV status for mice) with interaction was used, followed by post hoc Bonferroni's test. Statistical calculations were performed using GraphPad Prism 5.0. Data were expressed as the mean $\pm$ SEM. Significance was accepted at $p < 0.05$.

Results

As expected, high-fat-fed rats gained more weight than normal-fat-fed rats (see online suppl. Table 1; see www.karger.com/doi/10.1159/000509217 for all online suppl. material). RSV (4 mg/kg per day s.c.) did not affect weight gain. No significant difference was found in fed plasma glucose (online suppl. Table 1) or fed plasma insulin, triglyceride, and free fatty acid levels (online suppl. Table 2).

Since hypertension is a risk factor for restenosis [47] and studies have shown that RSV can decrease blood pressure [48], blood pressure was measured. Systolic blood pressure was not significantly different between the high-fat- and the normal-fat-fed groups (online suppl. Fig. 1). RSV did not affect blood pressure in either diet groups.

Because RSV has insulin-sensitizing effects, whole-body insulin sensitivity was assessed using the hyperinsulinemic-euglycemic clamp protocol. Glucose and insulin levels did not differ among groups (Fig. 1a, b). As expected, high-fat feeding impaired insulin sensitivity (the glucose infusion rate necessary to maintain the clamp) compared to normal-fat feeding (main effect of diet $p < 0.001$), and there was a significant diet and RSV treatment interaction ($p < 0.05$), showing that the effect of high-fat feeding was inhibited with RSV treatment (Fig. 1c). These results are in agreement with the findings of previous studies showing that RSV improves fat-induced insulin resistance [23, 24]. Despite the high-fat diet-induced insulin resistance found with the sensitive hyperinsulinemic-euglycemic clamp method, fasting (and postprandial) plasma insulin levels were not significantly different in rats fed a normal-fat diet compared to the high-fat diet, consistent with a previous study, where we used the same short-term feeding regimen in rats [6].

Analysis of the cross-sectional area of the injured carotids showed that high-fat diet significantly increased

($p < 0.05$) and RSV decreased neointimal area ($p < 0.01$). Importantly, there was a significant interaction between diet and RSV ($p < 0.01$), indicating that RSV counteracted the effect of fat (Fig. 2). Thus, RSV decreased neointimal formation in high-fat-fed insulin-resistant rats, whereas it did not appear to be effective in normal-fat-fed rats.

Previous studies by our group and others have shown that RSV affects SIRT1 expression and the phosphorylation (marker of activation) of AMPK and eNOS. Western blots of uninjured aorta obtained at the end of the hyperinsulinemic clamp showed that high-fat diet decreased SIRT1 expression and the phosphorylation of AMPK and of its target ACC, effects prevented by RSV. Unexpectedly, high-fat diet did not decrease eNOS phosphorylation. Although the ratio between p-eNOS and total eNOS (eNOS phosphorylation) was not significantly increased, p-eNOS (p-eNOS/tubulin) was increased by RSV (main effect $p < 0.001$). High-fat diet impaired insulin signaling and increased the activity of the inflammatory kinase IKK β , as indicated by the decreased phosphorylation of Akt and the decreased total I κ B α (degraded after phosphorylation by IKK β). RSV restored Akt phosphorylation and I κ B α levels (Fig. 3).

In injured carotids, immunohistochemistry revealed that RSV increased p-eNOS and p-VASP ($p < 0.01$), which is a downstream molecule of the NO/cGMP signaling pathway, and high-fat diet decreased p-VASP ($p < 0.05$) (online suppl. Fig. 2). There was no significant interaction between RSV and diet. We also measured plasma NO levels. Although plasma NO tended to be decreased by high-fat diet and to be increased by RSV in normal-diet-fed rats, these differences were not significant (online suppl. Table 3).

In order to examine the role of SIRT1 in the effect of RSV to decrease neointimal formation in high-fat-fed conditions, we first examined the effect of RSV in high-fat-fed SIRT1^{Y/Y} mice that have a catalytically inactive

Fig. 3. Western blots of aorta obtained from rats fed a normal- or high-fat diet. **a–f** Representative images and quantifications of Western blots of uninjured aorta at the end of the hyperinsulinemic clamp in rats. Y axes represent densitometric ratios, normalized to control. NFC, normal-fat control; HFC, high-fat control; NFR, normal-fat with RSV; HFR, high-fat with RSV. P-protein, phosphorylated protein; T-protein, total protein. See text for protein abbreviation. **a, b, c, e, f:** $n = 6$ /group; **d:** $n = 8$ /group. The representative eNOS blots were cut and pasted from the same blot because they were accidentally arranged out of order. * $p < 0.05$, ** $p < 0.01$ HFC versus NFC, $^{§§}p < 0.01$, $^{§§§}p < 0.001$ HFC versus HFR, * $p < 0.05$ NFR versus NFC (post hoc Bonferroni's test after a significant main effect or interaction was found).

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SIRT1 and in their high-fat-fed wild-type controls. RSV did not significantly affect weight gain and fed plasma glucose (online suppl. Table 4). RSV (~36 mg/kg per day orally) significantly decreased neointimal area, and there was no interaction between genotype and RSV treatment, showing that RSV decreased neointimal formation irrespective of genotype (Fig. 4).

Since SIRT1 may have effects that are independent of its catalytic function [39, 40], we also examined the effect of RSV in high-fat-fed heterozygous mice with a truncated SIRT1 protein (SIRT1^{+/-}), as homozygous SIRT1 knockout is lethal [41], and their high-fat-fed wild-type controls. No significant differences were found in weight gain and fed plasma glucose levels between groups (online suppl. Table 5). Again, RSV decreased neointimal area in both SIRT1^{+/-} and controls, that is, irrespective of genotype (Fig. 5). Collectively, our data of Figures 4 and 5 show that neither inactivation nor deletion of one copy of SIRT1 diminished the effect of RSV.

RSV is an established activator of AMPK and to examine the role of AMPK in the observed effects of RSV, we used AMPKα2-null mice. No significant differences were found in weight gain between high-fat-fed AMPKα2-null and wild-type mice (online suppl. Table 6). Fed plasma glucose levels were also not different between AMPKα2-null and wild-type mice, similar to the two other mouse models. However, all three genetically modified models display abnormalities in glucose homeostasis when challenged by tolerance tests [43–45]. In contrast with the two models of SIRT1 inhibition used, in AMPKα2-null and wild-type mice, the effect of RSV showed a positive interaction with genotype ($p < 0.05$). Indeed, RSV decreased neointimal area only in wild-type controls, and its effect was abolished in AMPKα2-null mice (Fig. 6).

In injured femoral arteries, immunohistochemistry showed that RSV significantly increased p-eNOS in SIRT1^{Y/Y} mice with catalytically inactive SIRT1 and their controls ($p < 0.01$) (online suppl. Fig. 3). RSV effect on p-eNOS was not significant in SIRT1^{+/-} mice ($p = 0.06$) or AMPKα2-null mice and their respective controls. RSV

had no significant effect on p-VASP in any of the three groups (online suppl. Fig. 3). Plasma NO appeared to differ by mouse genetic background since it was higher in AMPKα2-null mice and their wild-type controls (C57BL/6 background) than in SIRT1^{Y/Y} mice or SIRT1^{+/-} mice and their wild-type controls (Sv129xCD1 and Sv129 background, respectively). However, plasma NO was not significantly affected by RSV in any of the three groups (online suppl. Table 7).

Discussion

We have previously shown that insulin decreases neointimal area after arterial injury in an eNOS-dependent manner [11]. However, the vasculoprotective effect of insulin is greatly diminished in rats fed a high-fat diet [6]. Therefore, activating mechanisms that bypass vessel insulin resistance at the level of eNOS or are unrelated to insulin action may benefit patients with insulin resistance. In insulin-sensitive chow-fed rodents, we have shown that RSV decreases neointimal area in an eNOS-dependent manner [14], and previous studies have shown that RSV can increase insulin sensitivity in high-fat-fed mice [23]. Therefore, RSV may be more effective under high-fat feeding than normal-fat feeding conditions. One study in female mice has shown that RSV was effective at decreasing neointimal area after high-fat feeding by an estrogen receptor-mediated NOS-dependent mechanism [16]; however, the effects of RSV in high-fat- versus normal-fat-fed conditions were not compared. The present study in male rodents demonstrated that (1) unlike insulin, RSV effect to decrease neointimal area is more evident under conditions of high-fat than normal-fat diet in rats, and (2) RSV decreases neointimal area in high-fat-fed mice by a mechanism that is not mediated by the catalytic activity of SIRT1 and does not appear to involve transcriptional co-regulation by SIRT1 or eNOS activation but is dependent on AMPKα2.

The doses of RSV used were based on our previous studies in rats and mice [14]. After oral administration, the bioavailability of RSV is low (therefore higher oral than s.c. doses are needed); however, some of the metabolites of RSV are bioactive and may contribute to its effect [49]. We have previously shown that much higher doses given i.v. to rodents [24] yield RSV concentrations achievable by oral supplementation in humans [50].

In the present study, the effect of RSV to decrease neointimal area was not evident in rats fed a normal-fat diet, in contrast to a significant protective effect demonstrated

Fig. 4. The effect of resveratrol (RSV) on neointimal area in mice expressing a catalytically inactive SIRT1 and wild-type controls. Representative images (×200) (a) and quantification of neointimal area (b) of femoral artery cross sections measured at 28 days after wire injury in genetically modified mice expressing catalytically inactive SIRT1 protein (SIRT1^{Y/Y}) or wild-type (WT) littermates (Sv129xCD1 background) fed either high-fat diet (HFC) or RSV-supplemented high-fat diet (HFR). Numbers in parentheses represent mouse number. A main effect of RSV ($p < 0.05$) was found.

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in a previous study using chow-fed rats [14]. This discrepancy could be attributed to several factors. For instance, the normal-fat diet used in the present study contained 12% fat by calories, whereas the chow-diet used in the previous study contained 18% fat by calories [14]. Hence, the higher fat content may have magnified the effect of RSV on neointimal formation in the past study. In addition, different individuals performed the balloon arterial injuries in the two studies. Similarly, since the implantation surgery of osmotic pumps was also conducted by two investigators, the absorption rate of RSV from the subcutaneous tissue may have been different. Nevertheless, the current study shows that high-fat feeding accentuates (or unmasks) the effect of RSV to decrease neointimal growth, in parallel with the SIRT1 and AMPK activating, insulin sensitizing (Akt activation), and anti-inflammatory (IkB α increase) effects of RSV. Our previous study in injured carotids collected at the end of a hyperinsulinemic clamp did not show decreased AMPK activation in high-fat-fed rats; however, a decrease in AMPK activation may have been masked by the increase induced by injury [12]. The significant decrease in p-Akt in high-fat-fed rats is consistent with the tendency observed in our previous study, where vessel collection was also conducted under hyperinsulinemic conditions [12], unlike a study by other authors where uninjured aorta was collected under basal normoinsulinemic conditions, that is, in the absence of insulin stimulation [51]. In contrast to the latter study [51] that used a longer-term high-fat diet and to our previous study in injured carotids [12], we failed to show a significant decrease in eNOS activation by high-fat diet. Additionally, in the present study, RSV increased p-eNOS in uninjured aorta collected during hyperinsulinemia, consistent with another paper, where we evaluated the effect of RSV in injured carotids collected under basal normoinsulinemic conditions [14].

Since eNOS activation may be affected by injury and vessel type in addition to the experimental setting at the time of vessel collection, we also performed immunohistochemistry of the injured vessels. We found that p-VASP, but not p-eNOS was decreased by high-fat diet in

injured carotids collected under basal normoinsulinemic conditions. Thus, the high-fat diet-induced decrease in p-eNOS in injured carotids found in our previous paper may be due to collection of the injured vessels during hyperinsulinemia [12]. In addition, RSV increased p-eNOS and p-VASP irrespective of diet. Regardless of the marker of eNOS activation or activity and the vessel tested, the effect of RSV on the eNOS marker did not correlate with the effect of RSV on neointimal growth, suggesting that other mechanisms may underlie, at least in part, the beneficial effect of RSV to decrease neointimal growth under high-fat-fed conditions. Under normal-fat-fed conditions, eNOS activation by RSV was apparently not enough to decrease neointimal growth in the present study in contrast to our previous findings [14]. Notably, our findings regarding the effect of RSV on eNOS are inconsistent with this effect being mediated only by AMPK, as AMPK phosphorylation did not increase in normal-fat-fed rats.

Since in the present study RSV did not appear to have a significant effect under normal-fat feeding conditions, we focused on the mechanism of its effect under high-fat feeding conditions. Using genetic mouse models, the current study found that the effect of RSV to decrease neointimal area in the high-fat-fed mice was not mediated by the catalytic activity of SIRT1. We found that the effect of RSV is likely also not mediated via other effects of SIRT1. However, we cannot rule out that the non-deacetylase-mediated effects of the single copy of SIRT1 may be mediating RSV action. It is also possible that chronic inhibition of SIRT1 in these mouse models elevates compensatory pathways, which diminish the role of SIRT1 in neointimal formation. Nevertheless, our present results indicate that the effect of RSV to decrease neointimal area is likely not mediated by SIRT1 but by AMPK since the effect of RSV was abolished in AMPK α 2-null mice.

The effect of RSV on neointimal growth correlated with its effect on p-eNOS only in mice with catalytically inactive SIRT1 and their controls but did not correlate with its effect on p-eNOS, p-VASP, or plasma NO in the other groups. In particular, AMPK α 2-null mice and controls showed a marked difference in the effect of RSV on neointimal growth without any difference in the effect of RSV on eNOS activation markers. Thus, our results are consistent with another mechanism of AMPK-mediated effect of RSV on neointimal growth than eNOS activation. The limitation of our mouse study is that we did not perform vessel injury in mice fed a normal-fat diet; therefore, we could not correlate the effect of RSV on eNOS activation with that on neointimal growth in the absence of high-fat feeding. Also, because this study was focused

Fig. 5. The effect of resveratrol (RSV) on neointimal area in heterozygous SIRT1-null and wild-type controls. Representative images ($\times 200$) (a) and quantification of neointimal area (b) of femoral artery cross-sections measured at 28 days after wire injury in heterozygous SIRT1-null (SIRT1^{+/-}) or wild-type (WT) littermates (Sv129 background) fed either high-fat diet (HFC) or RSV supplemented high-fat diet (HFR). Numbers in parentheses represent mouse number. A main effect of RSV ($p < 0.05$) was found.

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on the effect of RSV, not on that of insulin, we did not determine whether the high-fat feeding-induced attenuation of the effect of insulin to decrease neointimal growth that we found in rats [6] is also confirmed in the mouse model.

AMPK has been shown to play an important role in the response to arterial injury. For example, the endothelial-specific overexpression of constitutively active AMPK α 1 in mice can decrease neointimal hyperplasia, as well as enhance re-endothelialization and endothelial progenitor cell mobilization after arterial injury [36]. AMPK α 2-null mice develop exacerbated neointimal area and intimal cell proliferation after arterial injury [37]. Unlike the aforementioned study in AMPK α 2-null mice, we did not find differences related to genotype in the absence of RSV treatment, which may be explained by the different diet (high-fat diet in our study vs. chow-diet in the other study) and/or vessel injured (femoral artery collected 4 weeks after wire injury in our study vs. carotid artery collected 2 weeks after wire injury in the other study) [37]. This latter study has shown that upregulation of NF κ B by AMPK α 2 deletion results in upregulation of p27^{Kip1}, a cyclin kinase inhibitor, thus increasing VSMC proliferation and intimal hyperplasia. Notably, in the present study, RSV restored I κ B α levels, consistent with downregulation of the NF κ B pathway. Thus, AMPK activation by RSV may decrease neointimal growth via an anti-inflammatory effect. Since the effect of RSV on neointimal growth paralleled its effect to increase AMPK and Akt phosphorylation as well as whole-body insulin sensitivity, we cannot exclude that increased vessel insulin sensitivity may have contributed to the effect of RSV via AMPK. However, we have unpublished data that in models of selective vascular insulin resistance, the vessel response to insulin treatment is diminished, but neointimal growth is unaffected in the absence of exogenous insulin.

The present study raises the question of how RSV activates AMPK, since SIRT1 did not play a causal role in the effect of RSV on neointimal growth and eNOS, which may activate AMPK [52], also does not appear to be involved. In our previous study, we have shown that not all AMPK activators decrease neointimal growth, and in particular, metformin, which activates AMPK via increasing the AMP/ATP ratio, is ineffective [12]. RSV can activate AMPK via a similar mechanism; however, studies using high doses of RSV (50–3,000 μ m) in myotubes show that it can also activate AMPK via inhibiting phosphodiesterases. More specifically, the resulting elevation of cAMP activates Epac1 [33], which can subsequently activate CaMKII [53]. In addition, RSV has pleiotropic effects on calcium signaling [54] and an increase in intracellular calcium [55] would activate CAMKII. CaMKII is a known eNOS kinase [56], and it can also activate AMPK via CaMKK β [33]. Thus, there is indirect in vitro evidence that RSV could activate both eNOS and AMPK via CaMKII. The known effect of CaMKII on neointimal growth appears to be isoform dependent as CaMKII δ was reported to aggravate neointimal hyperplasia [57, 58], while CaMKII γ was reported to decrease it [59]. In turn, in vivo exposure to RSV has been reported to either activate [60] or inhibit CAMKII [61], and the isoform targeted by RSV is unknown. Irrespective of how RSV activates AMPK, it does so in injured arteries [14], whereas metformin does not [12], and the present study shows AMPK activation is causal for RSV protection against neointimal growth, which may be mediated by the anti-inflammatory effect of AMPK (Fig. 7).

Further studies will have to be performed to disentangle the effect of CAMKII and AMPK and their relation-

Fig. 7. Scheme of the postulated effect of resveratrol to decrease neointimal growth in high-fat-fed rodents. RSV, resveratrol; CaMKII, calcium/calmodulin-dependent protein kinase II; AMPK α 2, AMP-activated kinase (α 2 isoform); IKK β , I κ B kinase β ; VSMC, vascular smooth muscle cell.

Fig. 6. The effect of resveratrol (RSV) on neointimal area in AMPK α 2-null and wild-type controls. Representative images ($\times 200$) (a) and quantification of neointimal area (b) of femoral artery cross sections measured at 28 days after wire injury in AMPK α 2-null (AMPK α 2^{-/-}) or wild-type (WT) littermates (C57BL/6 background) fed either high-fat diet (HFC) or RSV-supplemented high-fat diet (HFR). Numbers in parentheses represent mouse number. $p < 0.05$ WT-HFR versus WT-HFC and WT-HFR versus KO-HFR (post hoc Bonferroni's test after a significant interaction between RSV and genotype was found).

ship to eNOS in the effect of RSV to decrease neointimal growth. In summary, the present study demonstrated that RSV decreased neointimal area in rats fed a high-fat diet, a model resistant to the “anti-restenotic” effect of insulin. Second, RSV also decreased neointimal area in mice fed a high-fat diet, an effect not mediated via SIRT1 catalytic activity and likely also not by other effects of SIRT1 but dependent on AMPK α 2.

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Statement of Ethics

All procedures followed the Canada Council of Animal Care standards and were approved by the Animal Care Committee of the University of Toronto.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J.G., T.J.P., D.M.B., and H.Z. acquired and analyzed data. Y.M., P.S.D., and M.G.M. analyzed data. A.G. conceived the work. D.F.M., M.W.M., S.P.H., E.L.T., and V.W.D. contributed to the conception of the work. J.G. and A.G. drafted the work, and all the other authors revised it critically. All authors gave final approval and agreed to be accountable for their work.

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