Resveratrol inhibition of inducible nitric oxide synthase in skeletal muscle involves AMPK but not SIRT1

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The radical gas nitric oxide (NO) is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). This inducible (iNOS) member of the NOS family is barely expressed or not detectable under normal conditions. However, in response to bacterial endotoxins, inflammatory cytokines, or nutrient overload, iNOS cellular expression is markedly upregulated and generates NO at very high rates and for prolonged periods of time (36, 43). Although this high-output NO pathway probably evolved to protect the host from infection, growing evidence shows that it is responsible for deleterious effects (e.g., hypotension, organ injury) seen in normal host cells, thereby conferring to iNOS the protective/destructive duality inherent in all major components of the immune response (40). One detrimental effect of iNOS induction in inflammatory conditions is the development of insulin resistance, as reported after acute systemic endotoxemia (23, 24). Indeed, iNOS expression is also increased in adipose tissue, muscle, and liver of animal models of obesity, insulin resistance, and diabetes (17, 46, 52). Importantly, mice lacking iNOS are protected from both LPS (9) and obesity-linked insulin resistance (43), providing conclusive genetic evidence that iNOS is a major inflammatory mediator in those settings. Clinical studies also revealed an important link between iNOS expression in muscle and adipose tissues and the development of obesity-linked type 2 diabetes (37, 53).

Resveratrol (RSV), a natural polyphenol mainly found in grapes and red wine, is known for its potential health benefits, especially owing to its antimutogenic and antioxidant properties. RSV has also been reported to induce changes associated with calorie restriction, including longer life span and protection against age-related diseases such as cardiovascular, neurological, and metabolic pathologies (2). Indeed, it has been reported that RSV can prevent diet-induced obesity and improve insulin sensitivity in mice fed with a high-calorie diet (1, 29). Interestingly, RSV can also downregulate various proinflammatory mediators in macrophages and interfere with the NO pathway in both macrophages and cardiac muscle in vivo (19, 38, 54, 57).

The underlying mechanisms responsible for the metabolic and anti-inflammatory effects of RSV remain the subject of intense research. RSV is thought to exert some of its metabolic actions via SIRT1, a NAD+-dependent protein deacetylase and one of the seven mammalian orthologs of the yeast protein silent information regulator 2 (Sir2) (30). Indeed, RSV has been shown to mimic calorie restriction and increase life span in a Sir2-dependent manner, thus providing a molecular link for its effects on longevity (21, 34). However, whether RSV acts directly through SIRT1 is still controversial (14, 42). Another potential target of RSV is AMP-activated protein kinase (AMPK), a master metabolic regulator and a well-established modulator of lipid and carbohydrate metabolism (66). AMPK can be stimulated by different physiological processes, including conditions that increase the AMP/ATP ratio and calcium concentrations (35, 63). Insulin-sensitizing actions of pharmacologic agents currently used in the treatment of type 2 diabetes, such as metformin and rosiglitazone, are thought to be explained in part by AMPK-dependent activation of glucose metabolism and lipid oxidation in muscle and liver (16, 39). Importantly, we also reported that AMPK activators inhibit iNOS induction in skeletal muscle and adipose tissue as well as in macrophages, leading us to propose that this anti-inflammatory action of AMPK-activating drugs likely contributes to their beneficial effects on obesity-linked insulin resistance (45).
In the present study, we have tested whether RSV inhibits iNOS induction in insulin target tissues and cells and have further explored whether activation of SIRT1 and/or AMPK were involved in this anti-inflammatory action of the polyphenol. The data show that RSV robustly inhibits endotoxin-mediated iNOS induction in muscle and adipose tissues in vivo as well as cytokine/LPS-mediated iNOS induction in cultured muscle and adipose cells. Our results further indicate that the ability of RSV to blunt iNOS induction in myocytes is at least partly mediated by the activation of the AMPK but not SIRT1.

METHODS

Materials. AICAR was purchased from Toronto Research Chemicals (Toronto, ON, Canada). Interferon-γ and TNFα were from Fitzgerald (Concord, MA) and R&D systems (Minneapolis, MN), respectively. All cell culture solutions and supplements were purchased from Life Technologies, except for fetal bovine serum, which was purchased from Sigma (St. Louis, MO). Reagents for SDS-PAGE and immunoblotting were from Bio-Rad and Amersham. Rabbit polyclonal antibodies against acetyl-CoA carboxylase, AMPK (which recognizes both AMPKα1- and -α2), p-Thr172-AMPK, and PPARγ coactivator-1α (PGC-1α) were purchased from Cell Signaling Technology (Beverly, MA) and were used at 1:500 and 1:1,000 dilutions, respectively. Rabbit polyclonal iNOS and p-Ser79 acetyl-CoA carboxylase were purchased from Millipore (Lake Placid, NY) and were used at 1:500 and 1:2,500 dilution, respectively. Mouse monoclonal α-tubulin antibody was from Sigma. Horse radish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA) were used at 1:10,000 and 1:20,000 dilutions, respectively. ECL solutions were from Millipore (Etobicoke ON, Canada), GE Healthcare, Little Chalfont, UK. Densitometric analysis was performed with ImageQuant TL software (GE Healthcare, Little Chalfont, UK).

Cell culture. A line of L6 skeletal muscle cells and 3T3-L1 fibroblasts (kind gift of Dr. A. Klip, Hospital for Sick Children, Toronto, ON, Canada) were grown in α-minimum essential medium (α-MEM, 10% fetal bovine serum) or α-Dulbecco’s modified Eagle’s medium (α-DMEM, 20% calf serum) and differentiated into myotubes and adipocytes as previously described (3, 24). FAO hepatic cells (kind gift from C. Ronald Kahn, Joslin Diabetes Center, Harvard Medical School, Boston, MA) were grown and maintained in Roswell Park Memorial Institute medium (RPMI, 10% fetal bovine serum). To induce iNOS, L6 and 3T3-L1 cells were incubated with cytokine/LPS mixture (TNFα 10 ng/ml, IFNγ 10,000 U/ml, and LPS 10 μg/ml) and FAO cells with cytokines (TNFα 10 ng/ml, IFNγ 10,000 U/ml, and IL-1β 40 ng/ml). RSV was added at the same time as cytokines and/or LPS at different concentrations as indicated in the figure legends. After 24 h of treatment, the accumulation of nitrite in the incubation medium was used as an index of NO production. Nitrite concentration was measured spectrophotometrically using the Griess technique as previously described (3).

RNAi studies. Small interfering RNAs (siRNAs) were synthesized by Integrated DNA Technologies (Coralville, IA) and annealed according to the manufacturer’s instructions. The sequences of AMPKα1 siRNA and AMPKα2 siRNA used and the transfection protocol have been previously described (22). Six hours after transfection, cells were washed, and the medium was changed to fresh α-MEM supplemented with 2% FBS.

Animals. All animal handling and treatments were approved and followed the guidelines set by Laval University Hospital Research Center’s Animal Care and Handling Committee. Male C57BL/6 mice were purchased from Jackson Labs (Bar Harbor, ME). Mice were housed two to four per cage under a 12:12-h light-dark cycle in animal quarters at 22°C and allowed unlimited access to standard rodent food and water. Mice 8–10 wk old were pretreated with a single intraperitoneal injection of RSV (40 mg/kg body wt) or its vehicle (polypropylene glycol-saline, 50:50) 15 min prior to the intraperitoneal LPS (15 mg/kg body wt) or saline treatment. After 6 h of treatment, mice were anesthetized with isoflurane (3%) and exsanguinated by cardiac puncture. Blood and tissues were rapidly harvested and stored in liquid nitrogen until further processing.

Measurement of plasma and tissue nitrite and nitrate. Nitrite and nitrate levels in plasma, skeletal muscle (quadriceps), epididymal adipose tissue, and liver were measured by fluorometric spectrophotometry (47). Briefly, blood was collected in tubes containing EDTA and centrifuged for 10 min at 3,200 g to obtain plasma. Tissues were grounded in liquid nitrogen using a mortar. The tissue powder was resuspended in 5 volumes of Tris-EDTA buffer (20 mM Tris, pH 7.5, 10 mM EDTA) containing a protease inhibitor cocktail. Tissue lysates were centrifuged at 2,800 g for 20 min, and protein concentration of the supernatant was measured by BCA protein assay (Thermo Scientific). Plasma and tissue lysates were then centrifuged at 5,000 g (4°C) overnight in an Ultrafree-Microcentrifuge 10000 NWMW filter unit. Nitrate was reduced to nitrite using nitrate reductase and the NADPH regenerating system as described previously (47). The fluorescence was measured at λex 360 nm and λem 450 nm.

Protein extraction and western blotting. Fifty milligrams of quadriceps muscle, WAT, and liver tissues were pulverized with a pestle and mortar in liquid nitrogen, and the powder was homogenized in 6 vol (muscle and liver) or 4 vol (WAT) of homogenization buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10% glycerol, 1% Igepal CA-630, 10 mM NaF, 2 mM Na3VO4, 1 mM PMSF, and protease inhibitors). For Western blot analysis, 10–50 μg of crude protein lysates was solubilized in sample buffer and loaded on a 6–9% acrylamide gel and subjected to SDS-PAGE and transferred to nitrocellulose membranes, and immunoblotting was performed as described previously (23). Bands were detected by standard chemiluminescence and were scanned with the Microtek ScanMaster i800 or detected using an EC3 Imaging System (UVP, Cambridge, UK). Densitometric analysis was performed with ImageQuant TL software (GE Healthcare, Little Chalfont, UK).

Nuclear extracts and PGC-1α lysis acetylation assays. L6 nuclear extracts were prepared as previously described (62). Forty micrograms of nuclear extracts was immunoprecipitated using rabbit polyclonal PGC-1α-H-300 antibody from Santa Cruz Biotechnology (Santa Cruz, CA). PGC-1α acetylation and total levels were detected by immunoblotting using acetyl-lysine (Cell Signaling technology). Beverly, MA) and PGC-1α antibodies as previously described (48).

RNA extraction and RT-PCR. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions and stored at −80°C. After spectrophotometric quantification and verification of the total RNA quality, cDNA synthesis was carried out using a cDNA Synthesis Kit according to the manufacturer’s recommendations (Roche Applied Science, Laval, QC, Canada). cDNA was mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and a gene-specific primer and probe mixture (predeveloped TaqMan Gene Expression Assays, Applied Biosystems) in a final volume of 20 μl. The assays used were as follows: iNOS (Rn00561646_m1) and GAPDH as the housekeeping gene (Rn01775763_g1). All samples were run in triplicate on a Rotor Gene 3000 System (Montreal Biotech, Montreal, QC, Canada).

Statistical analysis. The effects of the treatments were compared by Student’s t-test or ANOVA analysis followed by Fisher’s or Tukey’s post hoc test using the JMP-7 program (JMP SAS Institute, Cary, NC). All data are presented as means ± SE. Differences were considered to be statistically significant at P < 0.05.
RESULTS

Effect of RSV on LPS-induced iNOS expression and NO production in vivo. As reported previously (23, 24), acute LPS challenge in mice, a well-established model of endotoxemia, markedly increased iNOS protein levels in skeletal muscle, adipose tissue, and liver (Fig. 1, A–C). Pretreatment with RSV significantly reduced iNOS expression in skeletal muscle and adipose tissue (Fig. 1, A and B) but not in the liver (Fig. 1C) of LPS-treated mice. We next evaluated whether RSV reduces NO production in vivo by measuring the accumulation of nitrite/nitrate (NOx) in different insulin-sensitive tissues and plasma of LPS-treated mice. LPS only modestly increased NO levels in insulin target tissues, since NO is also produced by constitutive endothelial NOS and neural NOS enzymes in these tissues. Nevertheless, RSV was found to significantly reduce NO production in muscle and adipose tissue but not in liver (Fig. 2, A–C) of LPS-challenged mice. The tissue-specific action of RSV likely explains the lack of significant effect of the polyphenol on plasma NO levels (Fig. 2D).

RSV decreases cytokine/LPS-induced iNOS expression and nitrite production in L6 myocytes and 3T3-L1 adipocytes. We next tested whether the anti-inflammatory effect of RSV on iNOS induction could be recapitulated in vitro using insulin target cell lines. As we previously reported (3, 23, 24), exposure of L6 myocytes and 3T3-L1 adipocytes to cytokines and LPS markedly induced iNOS, as shown by a robust induction of iNOS protein and marked NO production as detected by the accumulation of nitrite in the culture medium (Fig. 3, A and B). Addition of RSV caused a dose-dependent reduction of cytokine/LPS-induced iNOS protein induction and nitrite accumulation, reaching a maximal inhibition at 40 μM in both cell lines (70% in L6 cells and 80% in 3T3-L1 cells; Fig. 3, A and C). However, iNOS mRNA expression remained unchanged in L6 myocytes after RSV treatment (Fig. 3B). Since LPS-mediated iNOS induction in liver has been shown to be mediated mainly through the release of IL-1β by inflammatory cells (26, 41, 50), we treated FAO hepatic cells with IL-1β and not LPS along with TNFα and IFNγ, as opposed to muscle and adipose cells, to better mimic the in vivo conditions. As observed with liver of LPS-treated animals in vivo, RSV failed to decrease cytokine-induced iNOS expression and NO production in FAO cells when used at equimolar doses (Fig. 3D).

SIRT1 inhibitors failed to prevent RSV-mediated iNOS inhibition in L6 myocytes. We next explored the potential mechanisms of RSV action using L6 myocytes. Previous studies have shown that SIRT1 exerts anti-inflammatory effects in various cell types (10, 65), suggesting that the deacetylase could be a good candidate for mediating RSV action on iNOS (14). However, we found that three different SIRT1 inhibitors failed to prevent RSV-mediated inhibition of NO production and

Fig. 1. Effect of resveratrol (RSV) treatment on inducible NO synthase (iNOS) protein expression in insulin-target tissues of LPS-treated mice. C57BL6 mice were treated with RSV (40 mg/kg) or vehicle 15 min prior to injection with LPS (40 mg/kg) or saline. iNOS protein expression was evaluated in quadriceps muscle (A), white adipose tissue (WAT; B), and liver (C) by Western blotting, as described in MATERIALS AND METHODS (3 representative animals per group are shown). Data are expressed relative to the mean of LPS-treated group. Bars represent means ± SE of 5–6 mice per group. *P < 0.05 vs. saline-treated values; #P < 0.05 vs. values of respective LPS-treated group.
iNOS protein expression (Fig. 4, A and B). Similar results were obtained in 3T3-L1 adipocytes (data not shown). We next verified whether SIRT1 was activated by RSV in L6 myocytes by determining the acetylation status of PGC-1α, a well-established readout of SIRT1 activity (48). Surprisingly, RSV (30 μM) did not deacetylate PGC-1α, confirming that RSV-mediated iNOS inhibition is not dependent on SIRT1 activity in L6 myocytes. In addition, all three SIRT1 inhibitors significantly increased the basal acetylation status of PGC-1α, suggesting that the pathway was efficiently invalidated in the cells (Fig. 4C).

**AMPK partly mediates the inhibitory effects of RSV on iNOS induction.** Since AMPK is another target of RSV action (13, 66), and AMPK activation inhibits iNOS in insulin target cells (45), we next explored whether this metabolic sensor was involved in RSV action on iNOS in muscle cells. RSV activates the AMPK pathway in L6 myocytes, as revealed by increased phosphorylation of the AMPK catalytic α-subunit on Thr172 and of its downstream target acetyl-CoA carboxylase on Ser79 (Fig. 5A), which was also observed in skeletal muscle of RSV-treated mice (data not shown). To determine the role of AMPK in RSV-mediated iNOS inhibition, we used RNAi to knockdown endogenous AMPKα1/α2 subunits. Immunoblotting experiments indicated that transfection of AMPKα1/α2 siRNAs reduced total AMPKα expression by 50 ± 5% in L6 myocytes (Fig. 5B). Importantly, the ability of RSV to inhibit cytokine/LPS-induced iNOS induction and NO production was significantly blocked. In addition, using Compound C, an AMPK inhibitor, we confirmed that the inactivation of AMPK partially blunted the ability of RSV to inhibit NO production in L6 cells (Fig. 5C). We obtained a similar tendency in 3T3-L1 adipocytes, but the effect was not statistically significant (data not shown). These results indicate that RSV counteracts iNOS induction in muscle cells at least in part through AMPK activation, as illustrated in Fig. 5D.

**DISCUSSION**

It is now well established that obesity and type 2 diabetes are chronic inflammatory disorders and that immunoinflammatory mechanisms are involved in the development of insulin resistance (20, 44, 60). It is therefore critical to identify endogenous anti-inflammatory targets and develop compounds that exert their actions through activation of such pathways. Naturally found in grapes and other plants, polyphenolic compounds such as RSV have been identified as potential mediators of numerous health benefits (2). It has been established that iNOS plays an important role in the development of insulin resistance in both acute systemic inflammation (endotoxin challenge) (3, 9, 23, 24) and chronic high-fat feeding and genetic obesity (11, 17, 43, 64). Here, we used the acute LPS challenge model to investigate whether RSV treatment inhibits iNOS induction in

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**Fig. 2. Effect of RSV treatment on nitrite/nitrate (NOx) production in insulin target tissues and plasma of LPS-treated mice.** C57BL6 mice were treated with RSV (40 mg/kg) or vehicle 15 min prior to injection with LPS (40 mg/kg) or saline. NOx accumulation was measured in quadriceps muscle (A), WAT (B), liver (C), and plasma (D) by fluorescence spectrometry assay as described in MATERIALS AND METHODS. Data are expressed relative to mean LPS-treated values. Bars represent means ± SE of 5–6 mice per group. *P < 0.05 vs. saline-treated values; #P < 0.05 vs. values of respective LPS-treated group.
insulin target tissues and cells. We have found that RSV counteracts cytokine-mediated iNOS induction in vitro and in vivo. This effect was not due to SIRT1 activation but could rather be attributed to the stimulation of the AMPK pathway.

A complex network of inflammatory pathways and mediators synergistically interacts to promote iNOS induction. Interestingly, there is evidence that RSV can inhibit JNK (28, 61) and NF-κB pathways (54) and interfere with the production of factors such as TNFα (4, 18), IFNγ (7, 18), and IL-1β (4, 18, 58, 67), which are known to be involved in iNOS induction during inflammation. Recent studies have reported that RSV inhibits iNOS expression in LPS-treated macrophages (54, 57), microglial (32) and Kupffer cells (25). Consistent with these results, we show here that RSV inhibits iNOS induction in insulin target tissues of LPS-challenged mice and found that this anti-inflammatory effect of RSV can be reproduced in cytokine/LPS-exposed muscle and adipose cells in vitro. Surprisingly, hepatic iNOS induction in LPS-treated mice was not attenuated by RSV treatment, and this lack of action of the polyphenolic compound was also observed in vitro in FAO hepatocytes. Although RSV has been previously reported to inhibit oxidative stress and NO metabolites in the liver of rats, this was after 7 days of daily RSV (20 mg/kg ip) treatment before LPS injection (49). In the present study, RSV was given as a single dose (40 mg/kg) 15 min prior to LPS injection. Thus, longer or repeated treatments with RSV may be needed to inhibit iNOS in liver of LPS-challenged animals. Another possibility is that the mechanisms by which LPS and cytokines activate iNOS in liver are different than those elicited in muscle and fat. LPS-mediated iNOS induction in liver has been shown to be mediated mainly through the release of IL-1β by inflammatory cells (26, 41, 50), whereas in muscle and adipose tissue iNOS induction is mostly linked to TNFα and IFNγ inflammatory mechanisms (3, 24). Therefore, since greater activation of iNOS can be obtained by mimicking these in vivo conditions, we exposed hepatic cells to IL-1β and not LPS.
along with TNF\(\alpha\) and IFN\(\gamma\) as opposed to muscle and adipose cells. Interestingly, it has been reported that the proinflammatory effects of IL-1\(\beta\) may be resistant to RSV (27). Thus, it may be possible that the intracellular mechanisms by which IL-1\(\beta\) triggers iNOS induction are resistant to RSV action.

The physiological mechanism by which RSV exerts anti-inflammatory actions is still the subject of debate. Several studies suggest that SIRT1 is a key mediator of the beneficial effects of RSV (6). Indeed, SIRT1 has been shown to deacetylate and inhibit the tumor suppressor p53, resulting in reduced apoptosis in response to various stress stimuli (33, 56). SIRT1 can also deacetylate and inhibit NF-kB, which is a key component of the intracellular inflammatory response (51, 59) and required for iNOS induction by inflammatory cytokines (12). Furthermore, it was recently shown that SIRT1 overexpression prevents cytokine-mediated cytotoxicity, NO production, and iNOS expression in a \(\beta\)-cell line (RIN) (31). It was also observed that SIRT1 activation by either RSV or overexpression of SIRT1 prevented cytokine toxicity and maintained normal insulin-secreting responses to glucose in isolated rat islets. However, we found that three different SIRT1 inhibitors failed to reverse the effect of RSV on iNOS protein expression and NO production in L6 myocytes. Furthermore, the concentration of RSV used here was not sufficient to significantly deacetylate PGC-1\(\alpha\), suggesting that in our model RSV is not inhibiting iNOS through increasing SIRT1 activity. Accordingly, recent studies have shown that RSV is a relatively nonspecific SIRT1 activator. Indeed, whereas RSV was re-

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ported to increase SIRT1 activity in vitro using a substrate containing a covalently attached fluorophore, the polyphenol failed to activate SIRT1 when native peptides or full-length protein substrates were used (42). Taken together, these studies strongly suggest that SIRT1 is not a mediator of RSV action on iNOS and most likely cannot account for the anti-inflammatory effects of the polyphenol.

AMPK has been reported to be a key mediator of RSV metabolic actions (55). We have previously demonstrated that AMPK activators exert anti-inflammatory effects by inhibiting iNOS induction (45). We found that RSV activates AMPK in L6 myocytes, consistent with previous studies in other cell lines and skeletal muscle in vivo (8, 13, 55, 66). Importantly, we show here that combined AMPKα1/α2 knockdown in L6 myocytes significantly reduced the ability of RSV to decrease cytokine/LPS-induced iNOS protein expression and NO production, indicating that AMPK activation is at least partly responsible for RSV-mediated iNOS inhibition in muscle cells. These results were further confirmed using the AMPK inhibitor Compound C. Interestingly, our results show that the inhibitory effect of RSV on nitrite production was highly correlated with a reduction in iNOS protein content but not in iNOS mRNA as we have already observed with several AMPK activators (45), suggesting that protein turnover was affected. This is in line with previous reports indicating that both AMPK activation (5) and RSV (15) can interfere with protein synthesis via posttranslational mechanisms not yet fully elucidated. Therefore, further studies will be required in order to understand the exact mechanism by which AMPK activation regulates iNOS induction.

In summary, we found that RSV potently inhibits iNOS induction in insulin target tissues and cellular models through activation of AMPK but not SIRT1 (Fig. 5D). These data provide new insights on the mechanisms underlying the beneficial effects of RSV on immune-inflammatory disorders,
offering the possibility to exploit this novel mechanism for new therapeutic opportunities.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are reported by the authors.

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