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Does resveratrol improve insulin signaling in chronically ischemic myocardium?

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Abstract

Background—Resveratrol is a naturally occurring polyphenol believed to be cardioprotective. We previously demonstrated that resveratrol improves insulin signaling and glucose metabolism in liver and skeletal muscle of swine with metabolic syndrome. Although resveratrol has metabolic benefits in peripheral tissues, its effect on insulin signaling in ischemic myocardium (IM) is unclear. Therefore, we developed a clinically relevant swine model of metabolic syndrome and chronic myocardial ischemia to investigate the effects of resveratrol on insulin signaling in cardiac tissue.

Materials and methods—Thirteen male Yorkshire swine were fed a high-cholesterol diet for 4 wk then underwent surgical placement of an ameroid constrictor to their circumflex artery to induce chronic myocardial ischemia. The high-cholesterol control group was given no drug ($n = 7$). The experimental group was provided the same diet and received supplemental resveratrol (100 mg/kg/d) ($n = 6$). Tissue was harvested 7 wk after ameroid placement for western blot and histological analyses.

Results—In IM, there was no significant difference between the two groups in the insulin signaling markers studied. In nonischemic myocardium, there was a significant decrease in phosphorylated AMP-activated protein kinase α ($P = 0.021$) in the group treated with resveratrol; otherwise, there were no significant differences between the groups. Immunostaining for glucose transporter 4 and Periodic acid-Schiff staining for myocardial glycogen stores was similar between the groups.

Conclusions—Resveratrol has complex effects on glucose metabolism. Although prior studies demonstrated that resveratrol supplementation improves insulin sensitivity in peripheral tissues, in chronically IM, there are no significant alterations.

Keywords

Resveratrol; Chronic myocardial ischemia; Metabolic syndrome; Insulin signaling; Diabetes; Glucose metabolism

1. Introduction

Despite public health efforts emphasizing lifestyle modification and pharmacologic treatments focused on reducing hypertension and high cholesterol, coronary artery disease

(CAD) remains one of the leading causes of death worldwide. Cardiovascular disease is responsible for up to 38% of all deaths in North America and remains the leading cause of death in both men and women in the developed world [1]. This trend is principally linked to continued rising rates of obesity and diabetes in the western world [2]. It is evident that insulin resistance increases the risk for CAD even without hyperglycemia [3,4]. Thus, in addition to modifying other risk factors, an improved understanding of how insulin signaling may influence cardiac risk factors is critical in treating this growing population.

Resveratrol is a naturally occurring polyphenol found in abundance in many berries and in the grapes used to make red wine [5]. In France, compared with other developed countries, consumption of red wine is higher and rates of mortality from CAD are lower [5,6]. This phenomenon, termed the “French Paradox,” has led to increased interest in the benefits of red wine and specifically resveratrol. The reported advantages of resveratrol are vast and include benefits in cancer, inflammation, longevity, diabetes, and cardiovascular health [5,7–9]. The extent and mechanism by which resveratrol induces its cardioprotective effects remain unclear. Several studies have demonstrated the complex and diverse influence of resveratrol on metabolic pathways, including insulin signaling [5,8,10,11]. We have previously demonstrated that resveratrol mitigates risk factors that lead to the development of CAD in a large animal model [12]. This study also demonstrated the beneficial effect of resveratrol on inflammation and glucose transport. In another study, we demonstrated that insulin signaling and glucose metabolism were improved with supplemental resveratrol in liver and skeletal muscle in a swine model with metabolic syndrome [11].

The insulin signaling pathway is intimately involved with other pathways involved in cell repair, survival, and death. Furthermore, this pathway has been shown to mitigate tissue injury resulting from physiologic stress including ischemia and reperfusion injury [13]. Given the known cardioprotective effects of resveratrol and having demonstrated its positive effect on insulin signaling in peripheral tissue, we sought to further investigate whether resveratrol similarly improves insulin signaling in chronically ischemic myocardium (IM) in the setting of metabolic syndrome.

2. Materials and methods

2.1. Animal model and surgical interventions

Adult male Yorkshire miniswine (Parsons Research, Amherst, MA) were fed a high-cholesterol diet over an 11-wk period. The animals were given daily feedings of 500 g of a high-cholesterol diet providing 2248 kcal/d consisting of and 75% regular chow, 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, and 1.5% sodium cholate. The high-cholesterol control [HCC] group ($n = 7$) received no drug. The experimental group was fed the same diet and supplemented 100 mg/kg/d of resveratrol (ChromaDex, Irvine, CA) orally ([HCRV], $n = 6$). Feedings were observed to confirm complete consumption of food and supplement. After 4 wk of dietary modification, animals in both groups underwent surgical placement of a titanium (1.75–2.25 mm in diameter) ameroid constrictor (Research Instruments SW, Escondido, CA) on the proximal left circumflex coronary artery.

Anesthesia was induced with an intramuscular injection of ketamine (10 mg/kg). Animals were intubated endotracheally and ventilated mechanically at 12–20 breaths/min. General anesthesia was maintained with a gas mixture of oxygen at 1.5–2 L/min and isoflurane at 0.75%–3.0% concentration. Postoperative pain was controlled with a single dose of intramuscular Buprenorphine (0.03 mg/kg) and a 72-h Fentanyl patch (4 µg/kg). All animals received perioperative antibiotics, enrofloxacin 68 mg daily for 5 d. Aspirin 325 mg was also given to all animals for prophylaxis against thromboembolic events starting 1 d preoperatively and continued for a total of 5 d.

Seven weeks after ameroid placement, the animals were euthanized and the heart was harvested. Tissue samples from chronically IM in the left circumflex coronary artery territory and nonischemic myocardium (NIM) were rapidly frozen in liquid nitrogen for molecular studies or placed in 10% formalin for immunohistochemistry studies. All experiments were approved by the Institutional Animal Care and Use Committee of the Rhode Island Hospital. Animals were cared for in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals.

2.2. Western blots

Radioimmunoprecipitation assay buffer (Boston BioProducts, Worcester, MA) was used to isolate whole cell lysates from homogenized tissue. Myocardial samples from chronically ischemic territories and nonischemic territories were assessed separately. Total protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Forty micrograms of total protein were fractionated by 4%–12% Bis-Tris gels electrophoresis (NuPage Novex Mini Gel; Invitrogen, Carlsbad, CA). Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Each membrane was incubated overnight at 4°C with the following primary antibodies: retinol binding protein 4 (RBP4) (Abnova, Taipei, Taiwan); adenosine monophosphate (AMP)-activated protein kinase α (AMPK α), phosphorylated AMP-activated protein kinase α (pAMPK α) (Thr 172), insulin receptor substrate 2 (IRS2), phosphoinositide 3-kinase (PI3K), AKT, pAKT (Ser 473), forkhead box 01 (FOX01); phosphorylated forkhead box 01 antibodies (p-FOX01) (Ser 256), glycogen synthase kinase-3 β (GSK-3 β), phosphorylated glycogen synthase kinase-3 β (p-GSK-3 β) (Ser 9), glucose transporter 4 (GLUT4) (all from Cell Signaling Technology, Danvers, MA); phosphorylated insulin receptor substrate 2 (p-IRS2) (Ser 731), glucose transporter 1 (GLUT1) (Abcam, Cambridge, MA); PGC1 α (Cayman Chemical, Ann Arbor, MI). Membranes were incubated with the appropriate horseradish peroxidase-linked secondary antibody for approximately 1 h at room temperature (Jackson ImmunoResearch, West Grove, PA). The immune complexes were visualized with enhanced chemiluminescence, and the images were captured with a digital camera system (G-Box; Syngene, Cambridge, UK). All membranes were probed with glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology) to correct for protein loading. Lysates and western blots were repeated in duplicate.

2.3. Immunohistochemical analysis

Frozen tissue from chronically IM was sectioned (thickness 12 μ m) and fixed in 10% formalin for 10 min. Sections were blocked with 1% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature and incubated with anti-GLUT4 antibody (Cell Signaling Technology) overnight. Sections were incubated with DyLight 549-conjugated antirabbit antibody (Jackson ImmunoResearch) for 45 min and then mounted with Vectashield with 4',5'-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were captured at $\times 20$ magnification with a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan). The same exposure and brightness was used for all images.

2.4. Histology

Frozen sections from chronically IM underwent Periodic acid-Schiff staining by the Pathology and Histology Core Facility at Rhode Island Hospital. Images were captured at $\times 20$ magnification using the Aperio ScanScope technology (Vista, CA).

2.5. Data analysis

The western blot bands and immunohistochemical analyses were quantified with densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). Probability values (*P* values) for the western blot and immunohistochemical data are reported from a *t*-test of the densitometry arbitrary units. *P* values were considered significant if <0.05 . Western blot results are expressed as fold change $\pm$ standard error of the mean compared with HCC.

3. Results

3.1. Animal model

All animals included in the analysis survived the procedures and completed the study. Body mass index (BMI) was measured 4 and 11 wk after initiation of the diet. After 4 wk, there was no significant change in BMI when comparing the two groups (Fig. 1). However, after 11 wk, the BMI was significantly increased in the HCC group compared with the HCRV group ($P = 0.022$; Fig. 1). Also at 11 wk, 50 g of dextrose was administered and glucose levels were subsequently measured. In the group supplemented with resveratrol, there was a significant decrease in glucose levels 30 min after dextrose was administered ($P = 0.012$; Fig. 2).

3.2. Western blots

In the IM, there were no significant changes in markers of insulin signaling between the HCC and HCRV groups (Table 1). In the NIM, there was a significant decrease in pAMPK α ($P = 0.021$) in the HCRV group compared with the HCC group (Table 2). Otherwise, there were no significant differences in protein levels measured with western blotting between the two groups (Table 2).

3.3. Histology

Immunostaining of frozen-fixed sections from the IM for GLUT4 demonstrated no significant difference (Fig. 3) between the animal groups. Periodic acid-Schiff staining of IM for glycogen stores was also similar between the two groups (Fig. 3).

4. Discussion

In previous studies, we demonstrated that oral resveratrol supplementation significantly modified major risk factors for cardiovascular disease in a swine model of metabolic syndrome with chronic myocardial ischemia. Robich *et al.* demonstrated a decrease in serum cholesterol and markers of inflammation, including C-reactive protein, with supplemental resveratrol as well as improved glucose tolerance [7]. Burgess *et al.* showed that animals supplemented with resveratrol also had improved glucose metabolism in peripheral tissues through improved insulin signaling [11].

Our present study demonstrates that resveratrol supplementation in a swine model of metabolic syndrome does not significantly alter insulin signaling in chronically IM. The insulin signaling cascade begins when insulin binds to its receptor. This initiates tyrosine phosphorylation of IRS, which activates IRS. Of note, although IRS1 and IRS2 are both found within myocardium, IRS1 is more abundant in muscle, whereas IRS2 is more abundant in the liver. In our prior studies, we demonstrated that IRS1 is significantly increased in IM in the same swine model of metabolic syndrome with chronic myocardial ischemia supplemented with resveratrol [12]. However, IRS2 is not significantly increased in our present study. Tyrosine-phosphorylated IRS binds to and activates PI3K. PI3K

activates AKT. Again, we did not demonstrate significant changes in any of these intracellular markers responsible for insulin signaling.

In cardiac myocytes, GLUT1 and GLUT4 are mainly responsible for transmembrane glucose transport. GLUT4 accounts for 60% of total glucose carriers, whereas GLUT1 accounts for the remaining 40% [14]. Activated AKT leads to the translocation of intracellular GLUT4 to the plasma membrane, which results in improved glucose transport into the cell [15]. Insulin signaling also results in translocation of intracellular GLUT1 to the cell surface, though to a lesser degree than GLUT4. Thus, insulin-activated glucose transport is mediated mainly by GLUT4. In a prior study, we demonstrated that GLUT1 expression was significantly increased in IM of high-cholesterol fed pigs given resveratrol when compared with pigs fed a similar diet without resveratrol. In the same study, GLUT4 expression did trend upward in the IM of pigs fed a high-cholesterol diet with resveratrol compared with pigs fed a high-cholesterol diet alone; however, the change was not significant [7]. In our present study, there is a minimal increase in GLUT1 expression on western blot analysis but the change is not significant. We similarly found no significant change in GLUT4 expression between the HCC and HCRV groups in IM with western blotting and immunostaining. Although resveratrol supplementation may alter GLUT1 and GLUT4 expressions, in our model, these changes are not large enough to be consistently demonstrated in a significant manner.

It is widely demonstrated that diabetes globally worsens cardiac function and there is an increase in multivessel CAD and congestive heart failure in type 1 and type 2 diabetics. However, there is evidence that hyperglycemia may be cardioprotective during acute myocardial ischemia [16,17]. Cardiac energy mainly relies on adenosine triphosphate synthesized by mitochondrial oxidative phosphorylation. During a resting state, lipid oxidation contributes 60%–70% of the energy supply to the heart, while glycolysis provides the remainder [18,19]. During cardiac stress, there is an increased shift toward glucose utilization over fatty acids. In the setting of hypoxia with altered AMP:ATP ratios, AMPK activation results in increased GLUT1- and GLUT4-mediated glucose transport, which increases myocardial glucose uptake [17,20]. Chu *et al.* demonstrated that in a type I diabetic swine model with acute myocardial ischemia, there was decreased infarct size and an increase in cell survival proteins including endothelial nitric-oxide synthase, heat shock protein 27, nuclear factor-kappa B, and the mammalian target of rapamycin compared with nondiabetic swine. Despite being hyperglycemic and insulin deficient, it is postulated that these animals use noninsulin-dependent pathways to efficiently metabolize glucose in ischemic cardiac myocytes [16]. Similarly, the cardioprotective effects of resveratrol on IM may be independent of insulin signaling.

Although there are promising studies purporting the benefits of resveratrol and insulin signaling, there is also significant conflicting data in the literature. Brasnyo *et al.* found resveratrol to improve insulin sensitivity in type 2 diabetic humans. In platelets, the pAKT:AKT ratio was increased; thus they put forth that activating AKT may be one mechanism by which resveratrol improves insulin sensitivity [10]. Another study by Kang *et al.* [9] demonstrated that diet-induced obese mice had improved insulin action with resveratrol only in insulin-resistant conditions depending on the tissue studied. In this study, resveratrol improved AKT phosphorylation in liver and white adipose tissue of diet-induced obese mice, whereas in skeletal muscle, there was no improvement in insulin signaling. In the skeletal muscle, there was an increase in tyrosine-phosphorylated AMPK suggesting that resveratrol has a different effect on the skeletal muscle in these mice [9]. As mentioned already, in a prior study, our group demonstrated an improvement in insulin signaling in both liver and skeletal muscle with supplemental resveratrol [11]. However, our current results did not demonstrate similar changes in ischemic and nonischemic cardiac myocytes.

The second annual international conference on resveratrol in health convened in December 2012 and the expert panel recently published the *Summary of the Scientific Working Group Discussion*. In regard to resveratrol's effect on insulin sensitivity, they concluded that although there was sufficient evidence that resveratrol improves insulin sensitivity in mammalian experiments, there was no tissue-specific designation. In human experimental models, there were too many studies demonstrating no effect on insulin sensitivity with resveratrol. Thus, the panel was unable to make any conclusions or recommendations as to the effect of resveratrol on insulin signaling in humans. In fact, expert consensus concluded that there is not yet enough evidence to support the therapeutic effect of resveratrol to treat any specific condition [21]. We feel our experience with a clinically relevant large animal model further supports this summary.

4.1. Limitations

There are limitations to this study. Although porcine cardiac physiology closely resembles human physiology, alterations in physiology may differ among species. As this is a large animal model, we kept the number of animals in each group at a minimum while still allowing for the determination of statistical significance. Thus, interpretation of the results must be kept within this context. Also, the study was limited to 11 wk of diet modification and supplemental resveratrol. Other changes may have been noted if the data were analyzed at earlier and later time points. Furthermore, we are unable to comment on the differences in dose-mediated response of resveratrol, as supplementation was limited to a single relatively high dose for all animals in the HCRV group. Finally, although we did compare IM and NIM from the same treated animals, we did not include a time-matched control without any ischemic insult (sham procedure group). A sham procedure group may demonstrate different physiology even compared with the nonischemic territory of a heart, which may be globally stressed by an ischemic insult.

5. Conclusions

In this clinically relevant porcine model of metabolic syndrome and chronic ischemia, we demonstrate that supplemental resveratrol does not improve insulin signaling in IM and NIM. In similarly designed prior studies, we demonstrated that resveratrol modifies risk factors of metabolic syndrome and CAD [7,11]. There is significant promise that resveratrol does indeed contribute to metabolic changes in myocardium independent of insulin signaling. Also, there is evidence to suggest the effect of resveratrol varies substantially depending on the metabolic conditions and tissue type. Future studies should further explore the influence of resveratrol in other metabolic and molecular pathways including cell survival, apoptosis, and autophagy in cardiac myocytes.

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Fig. 1. BMI (kg/m²) measured at (A) 4 wk and (B) 11 wk after initiation of the respective diets. (A) There was no difference in BMI between the two groups when calculated 4 wk after initiation of their respective diets. (B) At 11 wk, there was a significant increase in BMI in the HCC group compared with the HCRV group ($P=0.022$). P values determined from a t -test.

Fig. 2.

Blood glucose measured at 11 wk 30 min after 50 g of dextrose given intravenously. There was a significant decrease in blood glucose in the HCRV group compared with the HCC group. *P* value determined from a *t*-test.

Fig. 3.
GLUT4 and intramyocardial glycogen staining in chronically IM.

Table 1

Protein expression in ischemic myocardium.

Targets	HCC	HCRV	<i>P</i> value
AMPK	1 ± 0.206	2.006 ± 0.481	0.067
pAMPKα (Thr 172)	1 ± 0.151	1.717 ± 0.406	0.11
IRS2	1 ± 0.165	0.987 ± 0.243	0.96
pIRS2 (Ser 731)	1 ± 0.076	0.883 ± 0.099	0.36
P13K	1 ± 0.099	1.291 ± 0.216	0.23
AKT	1 ± 0.083	0.975 ± 0.087	0.84
pAKT (Ser 473)	1 ± 0.167	1.011 ± 0.134	0.96
FOXO1	1 ± 0.0.156	1.098 ± 0.094	0.62
pFOXO1 (Ser 256)	1 ± 0.174	0.852 ± 0.059	0.47
GSK-3β	1 ± 0.090	0.842 ± 0.116	0.30
p-GSK-3β (Ser 9)	1 ± 0.190	1.172 ± 0.287	0.62
PGC1a	1 ± 0.127	1.319 ± 0.142	0.12
GLUT1	1 ± 0.089	1.053 ± 0.082	0.67
GLUT4	1 ± 0.098	0.767 ± 0.115	0.15

Protein expression listed as fold change ± standard error of the mean compared with HCC.

* Indicates significant difference between HCC and HCRV group (*P* value <0.05). *P* values determined from a *t*-test.

Table 2

Protein expression in non-ischemic myocardium.

Targets	HCC	HCRV	<i>P</i> value
AMPK	1 ± 0.164	0.967 ± 0.338	0.93
pAMPKα (Thr 172)	1 ± 0.140	0.532 ± 0.066	0.021 *
IRS2	1 ± 0.120	2.169 ± 0.637	0.077
pIRS2 (Ser 731)	1 ± 0.233	1.272 ± 0.238	0.43
PI3K	1 ± 0.116	0.998 ± 0.111	0.99
AKT	1 ± 0.287	1.051 ± 0.294	0.90
pAKT (Ser 473)	1 ± 0.180	1.329 ± 0.369	0.42
FOXO1	1 ± 0.168	1.097 ± 0.076	0.66
GSK-3β	1 ± 0.128	1.222 ± 0.143	0.28
p-GSK-3β (Ser 9)	1 ± 0.162	1.201 ± 0.271	0.51
PGC1α	1 ± 0.133	0.819 ± 0.117	0.36
GLUT1	1 ± 0.182	0.877 ± 0.147	0.64
GLUT4	1 ± 0.101	0.673 ± 0.120	0.058

Protein expression listed as fold change ± standard error of the mean compared with HCC.

* Indicates significant difference between HCC and HCRV group (*P* value <0.05). *P* values determined from a *t*-test.