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Resveratrol reduces chronic inflammation and improves insulin action in the myocardium of high-fat diet-induced obese rats

Resveratrol reduz a inflamação crônica e melhora a ação da insulina em miocárdio de ratos com obesidade induzida por dieta hiperlipídica

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ABSTRACT

Objective

To evaluate the effects of resveratrol on insulin signaling and inflammation pathway in the myocardium of high-fat diet-induced obese rats.

Methods

Thirty Wistar rats were divided into a control group (n=10, standard diet), obese group (n=10, high-fat diet), and obese supplemented with resveratrol group (n=10, 20 mg/kg/day) for eight weeks. An insulin tolerance test was performed at the end of the study period "0" (without insulin), 5, 10, 15, 20, 25, and 30 minutes after an intraperitoneal injection of insulin (2 U/kg). Body and epididymal adipose tissue were weighed. Fragments of the myocardium were extracted for Western blot analyses of insulin pathway and proinflammatory molecules.

Results

Resveratrol increased the rate of glucose disappearance, phosphorylation of the insulin receptor, insulin receptor substrate 1, and protein kinase B; and reduced expression of tumor necrosis factor alpha and of the molecules

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involved in proinflammatory signal transduction, namely Ikappa B kinase and nuclear factor kappa B complex. The results also suggest that higher insulin sensitivity and lower levels of proinflammatory molecules occurred regardless of weight and epididymal adipose tissue loss.

Conclusion

Resveratrol increases insulin action and reduces inflammatory molecules in the myocardium.

Indexing terms: Obesity. Resveratrol. Insulin resistance. Inflammation.

RESUMO

Objetivo

Avaliar o efeito do resveratrol sobre a via de sinalização da insulina e melhora do quadro inflamatório no miocárdio de ratos Wistar obesos induzidos por dieta.

Métodos

Ratos Wistar foram divididos em grupos: *controle (dieta padrão para roedores), obeso (dieta hiperlipídica) e obeso suplementado com resveratrol (20 mg/kg/dia), por 8 semanas (n=10). Ao final do período experimental, realizou-se o teste de tolerância à insulina, nos tempos 0 (sem insulina), 5, 10, 15, 20, 25 e 30 minutos após injeção intraperitoneal de insulina (2 U/kg). O peso corporal e o tecido adiposo epididimal foram mensurados. Fragmentos do miocárdio foram extraídos para análises da via da insulina e moléculas pró-inflamatórias através de Western blot.*

Resultados

Os resultados indicam que a intervenção com resveratrol aumenta a constante de decaimento da glicose, fosforilação do receptor de insulina, substrato do receptor de insulina e da proteína quinase B. A suplementação de resveratrol também reduziu os níveis proteicos do fator de necrose tumoral alfa e de moléculas envolvidas com a transdução do sinal pró-inflamatório (quinase indutora do kappa B e fator nuclear kappa B). Os resultados ainda sugerem que a melhora na sensibilidade à insulina e a redução das moléculas pró-inflamatórias ocorreram independentemente da perda de peso corporal e da redução do tecido adiposo epididimal.

Conclusão

A suplementação de resveratrol aumenta a sensibilidade à insulina, o que está relacionado à redução de fatores inflamatórios no miocárdio.

Termos de indexação: *Obesidade. Resveratrol. Resistência à insulina. Inflamação.*

INTRODUCTION

The rapid increase in the prevalence of obesity shows that environmental changes, such as inappropriate eating patterns and little physical activity, are determinants in the increase of the global obesity epidemic[1]. Obesity is a decisive risk factor for diseases such as diabetes Mellitus type 2, high blood pressure, and cardiovascular diseases[2,3]. Morbidly obese patients are at greater risk of sudden death and cardiac problems[4]. A change in insulin sensitivity is an important link between these diseases. Insulin resistance occurs in the heart of obese rats, leading to a reduction in the amount and translocation of the protein Glucose Transporter type 4 (GLUT4) to the sarcolemma[5]. Although the mechanisms that lead to insulin resistance have been widely studied in skeletal muscle, more studies on the cardiac tissue are necessary, along with possible pharmacological or non-pharmacological therapies.

Insulin is an anabolic hormone secreted by the pancreas in response to an increase in blood glucose[6]. Insulin action begins with insulin binding to a specific membrane receptor, called Insulin Receptor (IR)[7]. IR activation promotes tyrosine phosphorylation in various substrates, including Insulin Receptor 1 (IRS-1) and 2 (IRS-2)[8]. Phosphorylation of the IRS proteins exposes binding sites for another cytosolic protein called

Phosphatidylinositol 3-Kinase (PI3-K), activating it[9,10]. Phosphatidylinositol 3-Kinase activation increases serine phosphorylation of the protein kinase B (Akt). The Akt protein is a serine/threonine-specific protein kinase expressed in muscle tissue[11]. Through phosphorylation, the Akt protein is capable of activating many metabolic effects, such as glucose uptake as well as glycogen and protein synthesis.

Many molecules or molecular pathways of intracellular signal transduction can interfere with the insulin transduction pathway, possibly leading to insulin resistance. Of these, the literature mentions the importance of proinflammatory cytokines for the development of insulin resistance, especially IKappa B Kinase (IKK)/Nuclear Factor Kappa B (NF-κB) transcription factor pathway and Tumor Necrosis Factor alpha (TNFα)[12].

Some nutrients increase insulin action significantly[13,14], such as resveratrol (3,5,4'-trihydroxystilbene), a phytoalexin found in plants and some foods, including peanuts, mulberries, and grapes[15]. Resveratrol provides cardiovascular protection[16], inhibits platelet aggregation[17], reduces inflammatory factors[18], and improves glucose homeostasis[19]. The effects of resveratrol supplementation were investigated in insulin-resistant[20], high-fat diet-induced obese rats[21], and found to improve glucose homeostasis and whole-body insulin sensitivity. However, neither the proinflammatory pathway nor the myocardium was assessed. Hence, the present study assessed whether resveratrol supplementation reduces inflammation and improves the insulin signal transduction pathway in the myocardium of obese rats.

METHODS

Characterization of the animals and diet

Thirty four-week-old male Wistar rats weighing approximately 115 g were obtained from the *Universidade do Extremo Sul Catarinense* (Unesc) Laboratory Animal Facility. They were kept in 12/12-hour light/dark cycle at a temperature of 20ºC to 22ºC. After one week of adaptation to the new environment, the animals were first divided into two groups: a lean group fed with standard rodent chow (Nuvital Nutrientes S.A., *Colombo*, PR, Brazil) (carbohydrates: 70.0%; proteins: 20.0%; fats 10.0%, totaling 3.8 kcal/g) (n=10) called control group; and a group fed a high-fat diet (carbohydrates: 38.5%; proteins: 15.0%; fats 46.5%, totaling 5.4 kcal/g) (n=20) for eight weeks. Both groups were given water daily. Once the animals became obese, they were submitted to the Intraperitoneal Insulin Tolerance Test (ITT) to verify their insulin resistance. The obese animals were then subdivided randomly into two groups: high-fat diet-induced obese rats (obese, n=10) and obese rats receiving resveratrol supplementation (obese+resv, n=10).

All experiments were compliant with the principles and procedures established by the *Colégio Brasileiro de Experimentação Animal* (COBEA, Brazilian College of Animal Experimentation) and approved by Unesc's Research Ethics Committee under Protocol number 20/2011.

Resveratrol Supplementation Protocol

The supplemented animals received 20 mg/kg of resveratrol by gavage once a day for eight weeks. Resveratrol 100% pure was purchased from the company Pharma Nostra Ltda (*Rio de Janeiro*, Brazil).

Intraperitoneal insulin tolerance test

The test was performed at the end of the experimental period. Food was removed six hours before the test and the first blood collection corresponded to time "0" Next, insulin (2 U/kg of body weight) was injected intraperitoneally and blood samples were collected from a tail incision after 5, 10, 15, 20, 25, and 30 minutes to measure

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blood glucose. The constant of glucose disappearance during insulin tolerance test (k_{ITT}) was given by the formula $0.693/t_{1/2}$. The $t_{1/2}$ of glucose was given by Least Squares Analysis of the blood glucose level during the linear glucose disappearance phase.

Epididymal fat weight

The animals were sacrificed and the epididymal adipose tissue was removed and weighed by an analytical balance (Bel Engineering Ltda, *Piracicaba*, SP) with an accuracy of 0.001 g for comparing the groups. Body fat weight was expressed as a percentage of the body weight.

Tissue extraction and Western Blot

Twenty-four hours after the last supplementation, the rats were anesthetized with ketamine (50 mg/kg, Syntec; *Cotia*, SP) and xylazine (20 mg/kg, Syntec; *Cotia*, SP). Next, the abdominal cavity was opened and either saline (group without insulin (-), 0.1 mL) or regular insulin (group with insulin (+), 0.1 mL, 10^{-6}) was injected into the inferior vena cava. After the insulin (Humulin R; Eli Lilly, *São Paulo*, SP) injection, fragments of the myocardium were collected and immediately placed in a specific extraction buffer (1% Triton-X 100, 100 mM Tris, pH 7.4 containing 100 mM of sodium pyrophosphate, 100 mM of sodium fluoride, 10 mM of Ethylenediamine-tetraacetic Acid (EDTA), 10 mM of sodium vanadate, 2 mM of Phenylmethanesulfonylfluoride (PMSF), and 0.1 mg of aprotinin/mL) and homogenized by a Polytron® homogenizer (Polytron MR 2100, Kinematica, Switzerland). At the end of the extraction, the animals were sacrificed by guillotine decapitation. Triton X-100 was added to all samples, which were then kept in ice for 40 minutes. The homogenized samples were centrifuged at 11000 rpm for 30 minutes by the centrifuge Eppendorf 5804R (Eppendorf AG, Hamburg, Germany). The Bradford method determined the protein concentration on the supernatant. The proteins were then denatured by boiling at 100 ºC in a Laemmli buffer system containing 100 mM of Dithiothreitol (DDT).

Once the protein concentration was determined, the samples were loaded in Sodium Dodecyl Sulfate - Polyacrylamide Gel (SDS-Page) and separated by electrophoresis. The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane in the device Mini Trans-Blot® Electrophoretic Transfer Cell (BIO-RAD, Hercules, United States) and immediately blocked by a blocking buffer (5% albumin; 10 mmol/L of Tris; 150 mmol/L of NaCl; 0.02% Tween 20) for two hours to minimize binding between the antibodies and nonspecific proteins. The membrane was incubated for 12 hours with the primary antibodies anti-pIR$^{Tir1162/1163}$ and anti-pIRS1^{Tir971} acquired from Cell Signaling Technology (Beverly, MA, United States) and anti-pAkt^{Ser473}, anti-TNFα, anti-pIKKα, anti-NF-κB, and β-actin acquired from Santa Cruz Biotechnology (Santa Cruz, CA, United States). After the primary antibody incubation, the membranes were rinsed again with a basal solution for three 5-minute sessions and incubated with the secondary antibody conjugated to peroxidase for chemiluminescence (Thermo Scientific, Rockford, IL, United States). Band intensity and area were determined by reading the autoradiographs developed by densitometry using a scanner (HP® G2710) and the software Scion Image (Scion Corporation®).

Statistical analysis

The results were expressed as means ± Standard Error of the Mean (SEM) and analyzed by one-way Analysis of Variance (Anova) followed by the Bonferroni *post hoc* test. The significance level was set at 5% ($p<0.05$). The software Statistical Package for the Social Sciences (SPSS) version 17.0 for Microsoft Windows performed the statistical analyses.

RESULTS

The body and epididymal adipose tissue weights of the obese animals without and with resveratrol supplementation were similar (Figures 1A and 1B), but significantly higher than those of the lean animals. The insulin tolerance test showed that the obese group had lower insulin sensitivity than the lean group (p<0.05). However, resveratrol supplementation increased the insulin tolerance of the supplemented obese group in comparison with the non-supplemented obese group (Figure 1C).

The insulin receptor (Figure 2A), IRS1 (Figure 2B), and Akt (Figure 2C) of the control group given insulin were significantly more phosphorylated than those of the lean group given saline. IR, IRS1 and Akt phosphorylation in the obese group was lower than that in the lean group (p<0.05). However, phosphorylation of these molecules was significantly higher in resveratrol-supplemented obese rats than in non-supplemented obese rats (p<0.05).

The TNFα and NFκB (subunit p65) protein levels and IKKα phosphorylation were analyzed. Obese animals presented a significant increase in TNFα (Figure 3A), IKKα phosphorylation (Figure 3B) and NFκB (Figure 3C) protein levels when compared with lean animals, but supplemented obese rats presented lower values than non-supplemented obese rats (p<0.05).

DISCUSSION

Obesity is an important factor in the development of cardiovascular diseases and insulin resistance, and a decisive risk for diabetes Mellitus type 2[2,3]. The IKK/NF-κB inflammatory pathway and TNFα have a critical role in the development of insulin resistance in pathophysiological conditions like obesity[14]. High-fat diet-induced obese rodents presented a significant increase in insulin resistance and expression of inflammatory cytokines, such as IL-1β, IL-6, TNFα, and NF-κB[13]. In states such as

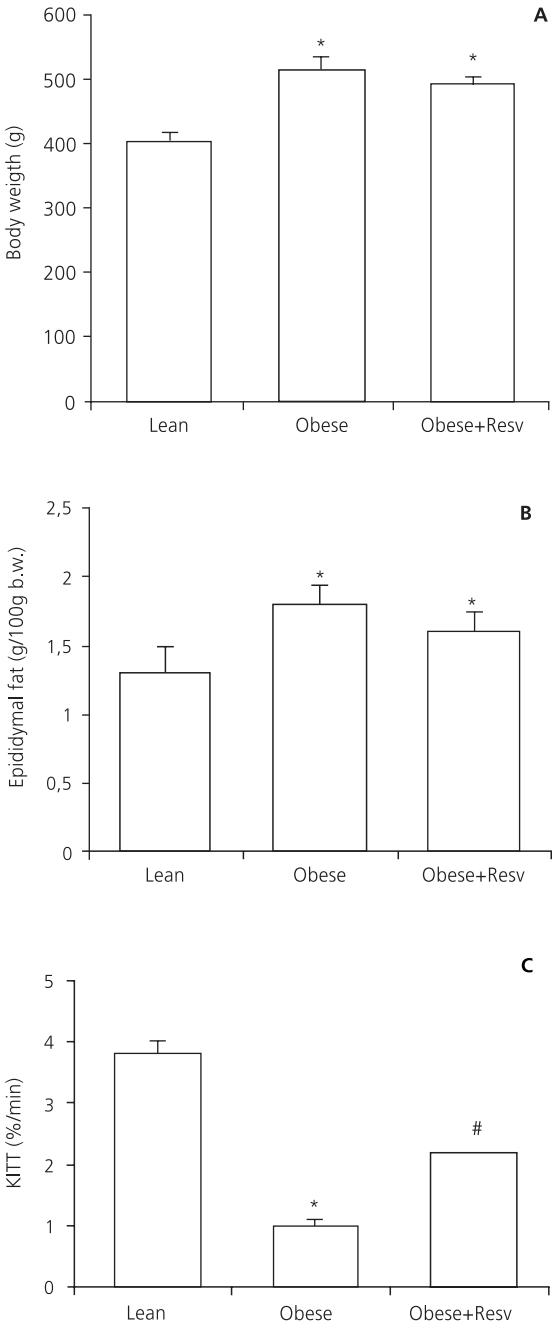

Figure 1. Analysis of body weight and epididymal fat, and rate of glucose disappearance (k_{ITT}) in the myocardium of lean rats, non-supplemented obese rats, and resveratrol-supplemented obese rats.

Note: Anova: *p<0.05 for non-supplemented obese rats and resveratrol-supplemented obese rats *versus* lean rats; #p<0.05 for resveratrol-supplemented obese rats *versus* obese rats. Body weight (A), epididymal adipose tissue weight (g/100g body weight) (B) and k_{ITT} (C). The results were expressed as arbitrary units. Bars represent means ± Standard Error of the Mean (SEM) of n=10 per group.

Source: *Universidade do Extremo Sul Catarinense* (Unesc). Laboratory of Physiology and Exercise Biochemistry. *Criciúma* (SC), Brazil, 2013.

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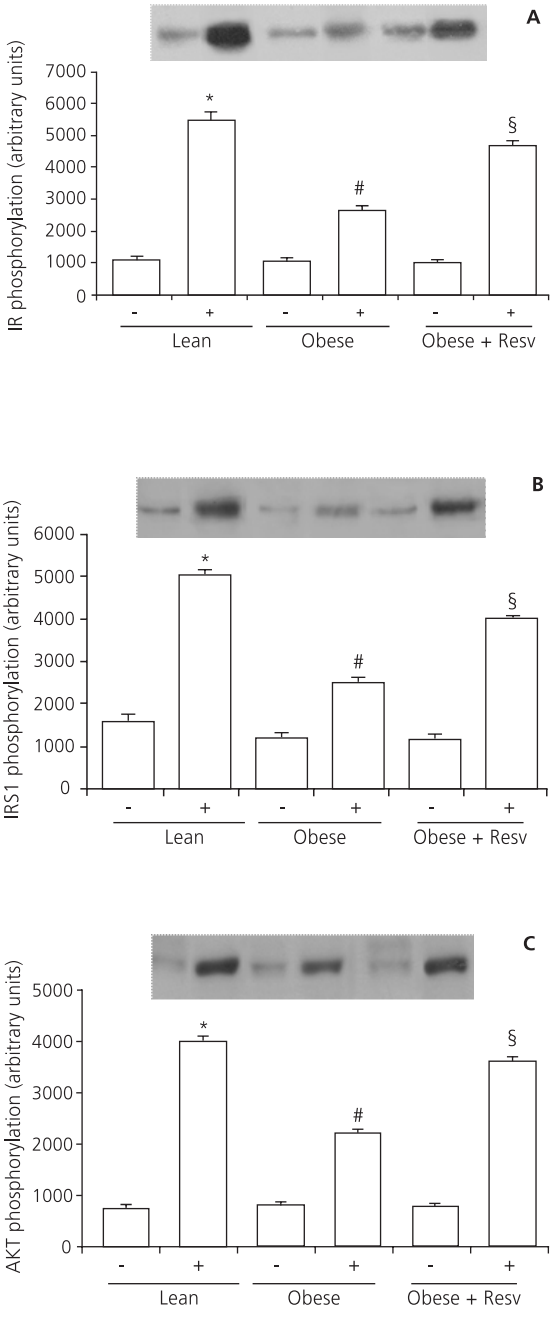

Figure 2. Insulin signaling pathway in the myocardium of lean rats, non-supplemented obese rats, and resveratrol-supplemented obese rats.

Note: Anova: $^{*}p<0.05$ for lean controls with insulin *versus* lean controls without insulin; $^{\#}p<0.05$ for obese rats with insulin *versus* lean controls with insulin; $^{\S}p<0.05$ for resveratrol-supplemented obese rats with insulin *versus* non-supplemented obese rats with insulin. IR (A), IRS1 (B), and Akt (C) phosphorylation. The results were expressed as arbitrary units. Bars represent means ± Standard Error of the Mean (SEM) of n=10 per group.

Source: *Universidade do Extremo Sul Catarinense*. Laboratory of Physiology and Exercise Biochemistry. *Criciúma* (SC), Brazil, 2013.

insulin resistance, cytokines such as TNFα activate IKKα and IKKβ by intermediate signaling pathways. This activation promotes the serine phosphorylation of IR and IRS1, reducing insulin signal transduction[22]. However, changes in inflammation levels and myocardial insulin signaling pathway require further investigation. Additionally, nutrients like resveratrol improve insulin sensitivity[19,23] and reduce inflammation and incidence of coronary diseases[16]. Thus, the study hypothesis was that resveratrol supplementation could benefit the myocardial insulin signaling pathway of obese rats. As expected, obese rats developed insulin resistance and presented higher levels of proinflammatory molecules in the myocardium, and resveratrol supplementation was capable of reversing these findings regardless of body weight and fat loss. Similar results were found in humans where a supplementation of 1g/day of resveratrol for 45 days improved insulin sensitivity, fasting glucose, and Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) regardless of body weight[24].

The resveratrol has also been shown to benefit the insulin signaling pathway[21,23]. Resveratrol supplementation increased IRS1 and Akt phosphorylation in the liver and soleus muscle of insulin-resistant rats, and reduced blood glucose and insulin[21]. The present study found similar results, that is, resveratrol supplementation increased IR, IRS1, and Akt phosphorylation in the myocardium and consequently, insulin sensitivity, as shown by the rate of glucose disappearance. Concordantly, resveratrol supplementation reduced hyperglycemia in an animal obesity model[25].

Regulation of the insulin pathway by resveratrol can be attributed to its ability to activate the protein Sirtuin 1 (SIRT1)[26] by inhibiting phosphodiesterase 4 with consequent Adenosine Monophosphate-Activated Protein Kinase (AMPK) activation[27]. SIRT1 activation helps to control glucose homeostasis by various mechanisms, such as regulation of insulin secretion[28], protection of pancreatic β-cells[29], IR modulation (which improves insulin resistance), inflammation

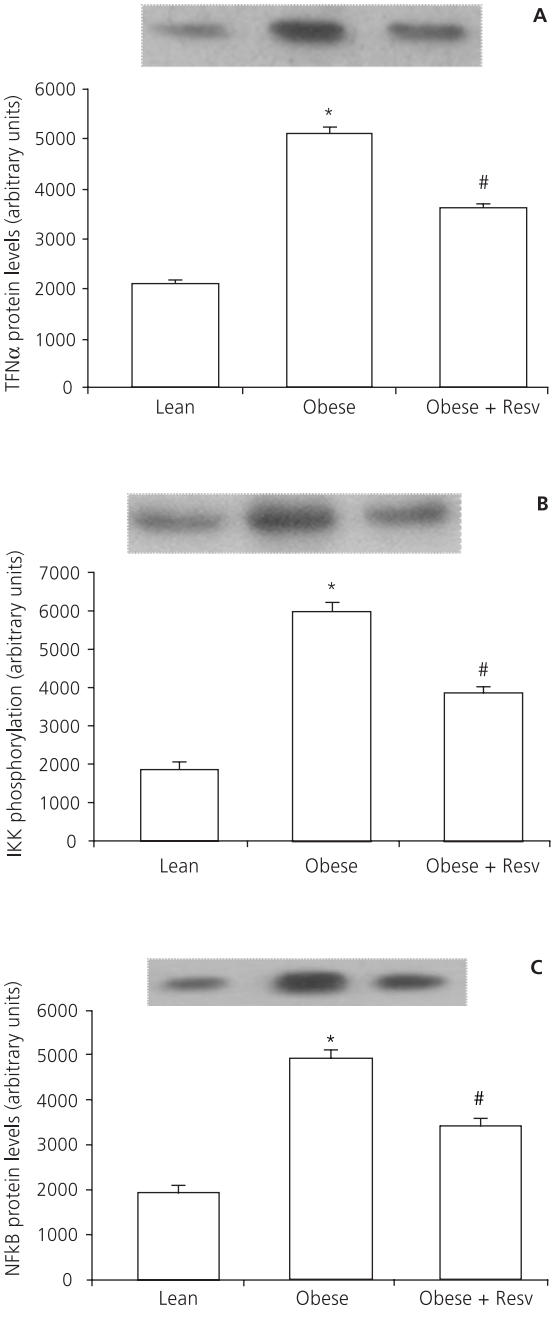

Figure 3. Protein and phosphorylation levels of enzymes involved in the proinflammatory signal transduction in the myocardium of lean rats, non-supplemented obese rats, and resveratrol-supplemented obese rats.

Note: Anova: $^{*}p<0.05$ for obese rats *versus* lean controls; $^{\#}p<0.05$ for resveratrol-supplemented obese rats *versus* non-supplemented obese rats. TNFα (A) and NFkB(p65) (B) levels and pIKKα (C) phosphorylation. The results were expressed as arbitrary units. Bars represent means ± Standard Error of the Mean (SEM) of n=10 per group.

Source: *Universidade do Extremo Sul Catarinense* (Unesc). Laboratory of Physiology and Exercise Biochemistry. *Criciúma* (SC), Brazil, 2013.

reduction, lipid mobilization, and adiponectin secretion[30]. The effects of 150 mg/day of resveratrol taken orally by obese men for 30 days were similar to those of calorie restriction, namely better insulin sensitivity and triglyceride levels, and activation of the AMPK/SIRT1 pathway in skeletal muscle[31].

The biochemical and molecular mechanism by which resveratrol reduces inflammation was not the focus of this study and deserves to be better clarified. However, this study clearly shows the effects of resveratrol on the levels of proinflammatory proteins. Moreover, Gonzales & Orlando[13] found that resveratrol acts on the NF-κB of adipocytes by inhibiting IKK, which prevents the translocation of NF-κB to the nucleus and consequently, reduces the transcription of inflammatory genes[13]. Other studies found that resveratrol reduces IKK phosphorylation in rats' liver[32] and expression of NF-κB and the cytokines IL-1β and IL-6 in fibroblasts[33]. In addition to its effect on the insulin pathway, SIRT1 can deacetylate and consequently, inhibit NF-κB, thereby reducing the transcription of proinflammatory genes[34]. SIRT1 activation by resveratrol also reduced the levels of proinflammatory molecules, such as JNK, IKK, and NF-κB[35].

Another explanation for resveratrol's ability to reduce inflammation may lie in its antioxidant nature[34]. Reactive oxygen species activate the molecules that regulate the inflammation pathway, including NF-κB, and promote the secretion of inflammatory mediators[36]. Furthermore, obese individuals have high levels of reactive oxygen species and impaired antioxidant defense system[37]. However, 20 mg/kg/day of resveratrol were capable of reversing the oxidative stress in the myocardium and aorta of obese rats[38]. Finally, the results are interesting because insulin's effects on the myocardium impacts heart survival[39]. These data evidence that resveratrol can be an effective non-pharmacological treatment to reduce inflammation and consequently, increase whole-body insulin sensitivity.

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Resveratrol treatment increases whole-body insulin sensitivity and the intracellular signaling of this hormone in the myocardium of obese rats, which apparently reduces the levels of proinflammatory cytokines.

CONTRIBUTORS

TF LUCIANO, SO MARQUES, BLS PIERI and DR SOUZA physiological and molecular analysis and article's drafting. FS LIRA analysis and data interpretation, article's drafting and critical revision of important intellectual content. CT SOUZA conception, design, analysis and data interpretation, article's drafting and critical revision of important intellectual content.

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