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Involvement of autophagy in the beneficial effects of resveratrol in hepatic steatosis treatment. A comparison with energy restriction.

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Key words: resveratrol, energy restriction, hepatic steatosis, autophagy, high-fat high-sucrose diet, rat

Abstract

Autophagy eliminates damaged cellular components. In liver it has been proposed that mediates the breakdown of lipid droplets. This study aimed to compare the involvement of autophagy and oxidative status in the effects of resveratrol and energy restriction as therapeutic tools for managing liver steatosis. In addition, potential additive or synergic effects were studied. Rats were fed a high-fat high-sucrose diet for 6 weeks and then divided into four experimental groups fed a standard diet: a control group (C), a resveratrol-treated group (RSV, 30 mg/kg/d), an energy restricted group (R, −15 %), and an energy restricted group treated with resveratrol (RR). Liver triacylglycerols (TG) were measured by Folch's method. TBARS, GSH, GSSG, GPx and SOD were assessed using commercial kits. Protein expression of beclin, atg5 and p62, as well as ratios of pSer555 ULK1/Total ULK1, pSer757 ULK1/Total ULK1 and LC3 II/I were determined by western blot. Energy restriction increased protein expression of beclin, atg5 and pSer757 ULK1/Total ULK1 and LC3 II/I ratios, and reduced protein expression of p62, thus indicating that it induced autophagy activation. The effects of resveratrol were similar but less marked than the hypocaloric diet. No differences were observed in oxidative stress determinants except for TBARS, which was decreased by energy restriction. In conclusion, resveratrol can reverse partially dietary-induced hepatic lipid accumulation, although less efficiently than energy restriction. The delipidating effect of energy restriction is mediated in part by the activation of autophagy, however the involvement of this process in the effects of resveratrol is less clear.

1. Introduction

Autophagy, a term first used by de Duve some decades ago [1], is an intracellular process in which cytoplasmic proteins and organelles are degraded by lysosomes [2]. This process serves to eliminate damaged cellular components and/or to maintain cellular energy homeostasis [3]. Three types of autophagy have been described so far: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy. In macro-autophagy (hereafter referred to as autophagy), organelles and cellular components are engulfed by a double membrane vesicle called autophagosome, which then fuses with lysosomes [4].

Although autophagy is a process that occurs constitutively in all eukaryotic cells, it can also be induced or impaired by different physiological and pathological signals (i.e. oxidative stress, starvation) in order to promote cell survival [4]. Moreover, oxidative stress is the situation in which the accumulation of reactive molecules (by-products derived from metabolic processes) exceeds the cellular capacity to repair biomolecule oxidation. The relationship between autophagy and oxidative stress is complex, because several regulatory mechanisms are involved. Some studies have suggested the existence of autophagic responses to oxidative stress situations, pointing towards mitochondrial-derived reactive oxygen species (ROS) as regulators of this response [5]. Moreover, in a study addressed in ULK1 knock-out mice, Li *et al*. [6] reported that the beneficial effects of resveratrol on oxidative stress were partially impaired by the deficiency of ULK1, thus demonstrating the involvement of autophagy in the anti-oxidant properties of this phenolic compound. Consequently, the relationships between autophagy and oxidative stress need further studies to be fully understood.

Hepatic steatosis is the more benign form of non-alcoholic fatty liver disease (NAFLD), a health alteration defined by a lipid accumulation greater than 5% of liver weight (chemically) or 5% or more hepatocytes showing triacylglycerol (TG) content

(histollogically). In liver, autophagy has been proposed as mediating the breakdown of hepatic lipid droplets in hepatocytes in a process called "lipophagy" [7, 8]. In this context, it has been observed that liver autophagic function is decreased in hepatic steatosis [7, 9].

Energy restriction, which consists in a reduction of 20-40% of total daily energy intake without malnutrition [10], has been reported to be an effective intervention for modulating oxidative stress [11]. Moreover, the positive effects of this nutritional approach on hepatic steatosis have been demonstrated in animal models [12-14] and in humans [15, 16]. In addition, this dietary intervention has been identified as a potent activator of autophagy [17]. As proposed by several authors, resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) could bring about the benefits of energy restriction without reducing caloric intake [18, 19]. Moreover, it has been reported that this phenolic compound is able to induce autophagy in a range of cell culture models [20-22]. However, to date is not clearly stablished whether the mechanisms of action of both energy restriction and resveratrol supplementation are the same or may be potentially synergistic.

In this scenario, the aim of the present study was to compare the involvement of autophagy and oxidative status on the effects induced by resveratrol administration and energy restriction as therapeutic tools for the management of liver steatosis previously developed by high-fat high-sucrose feeding. In addition, potential additive or synergistic effects were studied.

2. Material and Methods

2.1. Animals, diets and experimental design

The experiment was carried out using thirty-six 6-week-old male Wistar rats (Harlan Ibérica, Barcelona, Spain), and conducted in accordance with the University of the Basque Country´s Guide for the Care and Use of Laboratory Animals (Reference

protocol approval M20_2016_039). The rats were housed in individual polycarbonate metabolic cages (Tecniplast Gazzada, Buguggiate, Italy) and placed in a controlled air-conditioned room (22 ± 2 °C) with a 12-h light-dark cycle. After a 6-day adaptation period, the animals were fed a high-fat high-sucrose (HFHS) diet (OpenSource Diets, Lynge, Denmark; Ref. D12451) for 6 weeks. The diet provided 4.7 kcal/g of energy, being 45% of them as fat and 13% as sucrose. After this period, the animals were randomly distributed into four experimental groups (n=9) and fed for 6 additional weeks with a standard semi-purified diet (OpenSource Diets, Lynge, Denmark; Ref. D10012G) that provided 3.9 kcal/g, being 16% as fat, 64% as carbohydrates and 20% as protein: the control group (C), the resveratrol-treated group (RSV), the group submitted to energy restriction (R), and the group submitted to energy restriction and treated with resveratrol (RR). In the case of the animals on the C and RSV group, these had free access to food and water (*ad libitum*), while the animals in the R and RR groups were submitted to a mild energy restriction of 15% of total daily energy intake. In order to calculate the exact amount of food provided to the animals of these groups, the spontaneous food intake of the animals of the C group was taken into account. Based on previous experience of our group a dose of 30 mg resveratrol/kg body weight/day was selected, and provided as previously reported [23].

At the end of the whole experimental period (12 weeks), rats from the four experimental groups were sacrificed after an overnight fasting (12 hours) under anesthesia (chloral hydrate) by cardiac exsanguination. Livers were dissected, weighed, and immediately frozen in liquid nitrogen. All the samples were stored at -80 °C until further analysis.

2.2. Liver triacylglycerol content

Total liver lipids were extracted according to the method described by Folch *et al.* [24]. The lipid extract was dissolved in isopropanol, and the TG content was measured using a commercial kit (Spinreact, Barcelona, Spain).

2.3. Western blot

Liver samples (100 mg) were homogenized in 1000 μL of cellular PBS (pH 7.4), containing protease inhibitors (100 mM phenylmethylsulfonyl fluoride and 100 mM iodoacetamide). Homogenates were centrifuged at 800 *g* for 10 min at 4 °C. Protein concentration in homogenates was measured by Bradford method [25] using bovine serum albumin as standard.

Immunoblot analyses were carried out using 50 μg of liver extracts separated by electrophoresis in precast 4-15 % SDS-polyacrylamide gradient gels (Bio-Rad, Hercules, CA, USA) and transferred to PVDF membranes. The membranes were then blocked with 5 % caseine PBS-Tween buffer for 2 h at room temperature. Subsequently, they were blotted with the appropriate antibodies overnight at 4 °C. Specific antibodies were used to quantify the protein levels of 4-hydroxynonenal (4 HNE) (1:500), UNC-51-like kinase-1 (ULK1) (1:1000), sequestosome 1 (p62) (1:1000), autophagy protein 5 (Atg5) (1:1000) (Abcam, Cambridge, UK), mammalian homolog of yeast Atg 6 (Beclin 1) (1:1000) (Novus Biologicals, Littleton, CO, USA), microtubule-associated protein light chain 3 I and II (LC3 I/II) (1:1.000) (Cell Signaling Technology, Danvers, MA, USA) and beta-actin (β actin) (1:5000) (Sigma, St. Louis, MO, USA). Afterward, samples were incubated for 2 h at room temperature with polyclonal anti-mouse antibodies for p62 and β actin (1:5000) (Santa Cruz Biotech, Dallas, TX, USA) and anti-rabbit antibodies for 4 HNE, ULK1, Atg 5, Beclin 1 and LC3BI/II (1:5000) (Santa Cruz Biotech, Dallas, TX, USA), and the levels of the aforementioned proteins were

measured. After antibody stripping, in order to measure the phosphorylation status of different serine residues of ULK 1, the membranes were blocked, and then incubated with Serine 555 phosphorylated ULK1 (pSer555 ULK1) (1:1000) (US Biological, Salem, MA, USA) and Serine 757 phosphorylated ULK1 (pSer757 ULK1) (1:1000) (Cell Signaling Technology, Danvers, MA, USA). The bound antibodies were visualized by an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) and quantified with a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Specific bands were identified by using a standard loading buffer (Precision Plus protein standards dual color; Ref. 161-0374 Bio-Rad).

2.4. Oxidative stress determinants

Lipid peroxidation was determined spectrophotometrically by measuring the formation of thiobarbituric acid reactive species (TBARS) in liver homogenates using a commercial kit (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI, USA). The TBARS concentrations on the samples were calculated using a standard curve obtained with malonaldehyde (MDA).

GSH and GSSG amounts were colorimetrically assessed by using a commercial kit (Glutathione Colorimetric Assay Kit, BioVision Incorporated, Milpitas, CA, USA). The amounts of both GSH and GSSG were calculated using a standard curve. In order to determine the redox index, the ratio GSH:GSSG was calculated.

Glutathione peroxidase (GPx) level was also measured by using a colorimetric commercial kit (Glutathione Peroxidase Activity Colorimetric Assay Kit, BioVision Incorporated, Milpitas, CA, USA). The GPx amounts in the samples were determined using a standard curve obtained with NADPH.

Finally, superoxide dismutase (SOD) was assessed spectrophotometrically, using a commercial kit (Superoxide Dismutase Activity Assay Kit, BioVision Incorporated, Milpitas, CA, USA).

2.5. Statistical analysis

Results are presented as mean ± SEM. Statistical analyses were performed using SPSS 21.0 (SPSS, Chicago, IL, USA). All the variables were normally-distributed according to the Shapiro-Wilks test. Data were analyzed by one-way ANOVA followed by the Newman-Keuls *post hoc* test. Significance was assessed at the $p<0.05$ level.

3. Results

At the end of the experimental period (12 weeks), the body weights of the animals submitted to energy restriction (R and RR), were significantly lower than those found in the C group. Resveratrol administration did not induce significant changes on this parameter neither under standard feeding conditions nor under energy restriction Regarding liver weight, no differences were observed among the four experimental groups. All these results have been previously reported by our group [13].

As previously reported, when rats were fed a diet rich in fat and sucrose, a significant increase in liver TG was induced. In fact, those animals showed a 5.4% TG, an amount as is considered a steatosis [13]. This cohort of rats fed the high-fat high sucrose diet for 6 weeks, was distributed into four experimental groups and submitted to the treatments described in the Material and Methods section. As shown in Figure 1, after 6 weeks of treatment, lower TG values were found in the three treated groups in comparison with the C group ($p=0.03$ in RSV group, $p=0.0002$ in R group and $p=0.0004$ in RR group). In the case of the groups submitted to a mild energy restriction

(R and RR), lower TG values were found compared with the RSV group (p=0.003 in R group and p=0.005 in RR group), with no differences between them (Figure 1).

3.1. Western blot analysis in liver

In order to analyze the effects induced by the treatments on autophagy, the expression and activation of different proteins were analyzed. Phosphorylation ratios of different ULK1 serine residues were measured. Regarding pSer555 ULK1/Total ULK1 ratio, RSV and RR groups showed the highest values, which were significantly higher than those found in the C group (p=0.040 and p=0.007, respectively), with no relevant differences between both groups. As far as R group is concerned, a tendency towards higher phosphorylation ratio (+54.9%; p=0.086) was found when compared with the C group (Figure 2A). Regarding pSer757 ULK1/Total ULK1 ratio, RR group was the only group showing significantly lower phosphorylation ratio when compared with the C group (p=0.022). In the case of R group, a trend towards a lower phosphorylation ratio was observed when compared with the C group (-10.0%; p=0.078) (Figure 2B).

Beclin1 protein expression was significantly increased in R and RR groups when compared with the C group (p=0.044 and p=0.050 respectively), without statistical differences between them. In the case of RSV group, a trend towards greater protein expression was observed when comparing with the C group (+36.6%; p=0.072) (Figure 3). When Atg5 protein levels were measured, higher values were found in the energy restricted groups (R and RR) in comparison with the C group (p=0.042 and p=0.011), with no statistical differences between them. In the case of RSV, once again a tendency towards higher protein levels was found when comparing with the C group (+53.0%; p=0.10) (Figure 4A).

In the case of the LC3 II/I ratio, higher values were found in R and RR groups when compared with the C group (p=0.005 and p=0.009, respectively), without statistical differences between them. When comparing the ratio values of C and RSV groups, significant changes were not appreciated, but a trend towards a greater ratio was found in the RSV group (+36.1%; p=0.076) (Figure 4C). Finally, lower p62 levels were observed in R and RR groups when compared with the C group (p=0.016 and p=0.028), with no difference between them. Again in this case, there was a trend towards higher values was observed in RSV group (-19.8%; p= 0.088) (Figure 4B).

3.2. Oxidative stress determinants

Decreased hepatic TBARS formation was observed in the three treated groups (RSV, R and RR) compared with the C group (Table 1). Among the treated groups, when comparing the groups submitted to a mild energy restriction (R and RR) with the group treated with resveratrol (RSV), a trend toward lower values was observed in the R group (p=0.07), while in the case of RR group a significant reduction was observed in this parameter (p=0.016). No difference was found between both restricted groups (Table 1).

No differences were observed in either the levels of total glutathione or the GSH:GSSG ratio among the four experimental groups. Similarly, GPx remained unchanged, although a trend towards increased values was observed in R and RR groups when compared with the C group (p=0.09 and p=0.10 respectively). As far as SOD is concerned, no differences were appreciated in its activity among the four experimental groups (Table 1). Finally, no changes in 4 HNE protein levels were observed among the four groups (Figure 5).

4. Discussion

As indicated in the Introduction section, several studies have proposed that resveratrol acts as an energy restriction mimetic agent [18, 19, 26-28]. Nevertheless, several authors who have analyzed actions of this polyphenol other than those on fatty liver, have suggested that the mechanisms underlying the effects of resveratrol and energy restriction are not always the same [19, 29-32]. Taking into account that compliance scarcity is one of the main limitations of energy restriction interventions in humans (specially in the long-term), in this study an energy restriction degree lower than the commonly used 20-40% reduction, accompanied by the administration of resveratrol, was selected as anti-obesity strategy because we consider that the adherence to this protocol can be easier.

In the present study, both resveratrol and energy restriction played a beneficial role because they were able to reduce hepatic steatosis previously induced by high-fat, high-sucrose feeding, although the effect was greater in the case of energy restriction. A previous study carried out in our laboratory found that the effects of resveratrol and energy restriction on metabolic processes involved in liver TG metabolism (fatty acid uptake, fatty acid synthesis, TG esterification, fatty acid oxidation and TG export) were very similar, but not exactly the same. Thus, both dietary strategies decreased *de novo* lipogenesis and fatty acid uptake from blood stream, and increased fatty acid oxidation and liver TG delivery, but only energy restriction reduced TG assembly [13]. The present work focuses on autophagy, a process that limits hepatocyte lipid accumulation and mediates the breakdown of TG stored in lipid droplets. It has been proposed that a decrease in fusion efficiency between autophagosomes and lysosomes, due to membrane lipid alterations in response to high-fat diet, might underlie the inhibitory effect of diet-induced steatosis on autophagy, thus contributing to liver steatosis [33]. By contrast, energy restriction, a tool commonly used to treat fatty liver, stimulates

autophagy through the regulation of mTOR and AMPK [4, 34]. Moreover, it has been proposed that the progression of NAFLD to non-alcoholic steatohepatitis (NASH) is closely associated with impaired autophagy flux, while restoring autophagy balance improves NAFLD [8].

With regard to resveratrol, the involvement of increased autophagy in the beneficial effects of this bioactive molecule has been widely documented in cardiovascular diseases, cancer and neurodegenerative diseases [35, 36]. Moreover, the participation of autophagy in the anti-steatotic effect of resveratrol has been shown, but the number of studies reported so far is low and the experimental designs of these studies are different from that performed in the present work.

In order to analyse the involvement of autophagy in the beneficial effect of resveratrol on liver steatosis, the expression of proteins involved in the three major phases of autophagy, which are the initiation phase, the nucleation phase and the elongation/closure phase, were measured by western blot in the present study.

As far as the initiation phase is concerned, nutrient availability status regulates autophagy through ULK1. Mammalian target of rapamicyn complex 1 (mTORC1) and AMP protein kinase (AMPK) can modulate ULK1 activity by the phosphorylation of different serine residues [37]. Phosphorylation in serine 757 is mediated by mTORC1 action and exerts inhibitory effects on autophagy. Although reduced values of the pSer 757 ULK1/Total ULK1 ratio were observed in treated groups when compared with the control group, statistical significance was only reached in the group in which energy restriction and resveratrol were combined. Taking into account that in energy restriction states when nutrient availability is limited, and so mTORC1 is inhibited, the trend towards reduced values observed in the R group was to be expected. The decrease may

not have reached statistical significance because the applied energy restriction degree (-15%) was lower than usual (-20-40%).

Serine 555 phosphorylation is mediated by AMPK activation, which in situations of limited nutrient availability phosphorylates ULK1 and also inhibits mTORC1 in order to activate autophagy [37, 38]. In this regard, greater pSer 555 ULK1/Total ULK1 ratios were observed in the three treated groups (RSV, R and RR), although in the case of R group this increase was only a trend. These results are in good accordance with increased AMPK phosphorylation and thus AMPK activation induced by resveratrol in those rats, which we previously reported [13].

As far as the nucleation phase is concerned, the protein levels of Beclin1 were assessed. It has been reported that in situations of nutrient availability Bcl-2 binds Beclin1, thus inhibiting autophagy. By contrast, when nutrient availability is reduced, the Bcl-2/Beclin1 complex dissociates, thus activating autophagy [39]. Accordingly, in the present study rats submitted to mild energy restriction showed greater Beclin1 protein amounts, suggesting that this intervention was effective in promoting the nucleation phase. In the case of resveratrol administration, the trend towards greater Beclin1 protein amounts (36.6 % increase) is in accordance with the energy restriction mimetic role proposed for this compound, although the measured values were lower than those of energy restriction.

In the case of the elongation phase, Atg5 and p62 proteins, as well as the LC3BII/I ratio were measured. Atg5 is usually found conjugated with Atg12, and it is an important mediator in the early stages of the autophagosome formation [40]. This protein was significantly increased in both restricted groups (R and RR) and resveratrol-treated rats only showed a trend towards higher levels (53 % increase). With regard to LC3, the

conversion of the soluble form into the autophagosome-related lipidated form was studied by measuring LC3II/I ratio [41]. Lipid conjugation of LC3 promotes the closure of the autophagic double-membrane vacuole [42]. The higher values observed in the restricted groups (R and RR) suggest that the mild energy restriction used in the present study was able to effectively induce this conversion. As in the case of other proteins, resveratrol tended to activate this phase (36.6 % increase).

Finally, the protein p62 is able to bind ubiquitinated proteins and interact with LC3 in directing the autophagic process. During autophagy, p62 is degraded, and for this reason, its decrease is widely used as a marker of the autophagic flux [43, 44]. Changes in the expression of this protein reveal that, in those groups submitted to a mild energy restriction (R and RR), the autophagic flux was increased. This effect was discrete in resveratrol-treated rats, which only showed a trend towards decreased p62 values (19.8% decrease).

The results from the present study are in agreement with those reported by other authors using different experimental designs [14, 45]. In these studies increased Beclin1 and/or LC3II protein expression and decreased p62 protein expression were observed in liver from mice fed a methionine/choline-deficient diet or rats fed a high-fat diet, both treated at the same time with resveratrol, when compared with the control group. The main difference between the results reported by these authors and those found in our study is that while in the former, changes induced by resveratrol were statistically significant, in our study only statistical trends were observed. This difference can be explained in two ways. The first one is that in the reported studies, resveratrol was administered at the same time as liver steatosis-inducing diets, and treated groups were then compared with mice fed diets that inhibit autophagy. That is to say, they analyzed

the involvement of autophagy in the preventive effect of resveratrol on liver steatosis. By contrast, in the present study, rats were fed a high-fat high-sucrose diet in order to induce liver steatosis and then switched to a standard diet. Thus, resveratrol supplementation took place under normal feeding conditions and treated groups were compared with a control group fed a standard diet that did not impair autophagy. In other words, we analysed the involvement of autophagy in the positive effect of resveratrol for liver steatosis treatment. A second reason is that in the reported studies high doses of resveratrol were used (100 and 200 mg/kg body weight/d). The dose in the present study was lower (30 mg/kg body weight/d). This fact suggests that a dose-response effect of resveratrol on autophagy cannot be discounted.

The involvement of autophagy in the beneficial effects of resveratrol on liver steatosis was also described by Li *et al*., [6]. These authors showed that resveratrol was less efficient in ULK1 knock-out mice than in wild type mice. The authors explain that their study was devoted to analysing the therapeutic role on NAFLD instead of its preventive role against established NAFLD (as in the vast majority of the studies reported). Nevertheless, the experimental design was different from that used in the present study. Li *et al*. used a high-fat diet for 9 weeks in order to develop liver steatosis and then they treated mice with 50 mg resveratrol/kg body weight/d for 4 additional weeks, by maintaining the same high-fat feeding conditions. By contrast, we have used a high-fat high-sucrose diet for 6 weeks and then rats were switched to a standard diet supplemented with resveratrol (for 6 weeks more).

Taking together our results and those reported in the literature [6, 14, 45], it can be proposed that the involvement of autophagy in the beneficial effects on liver steatosis are clearer when stressing feeding conditions (i.e. high-fat diet) are present.

Nevertheless, further studies are needed to confirm this hypothesis. It is important to emphasize that the effects produced by energy restriction on liver TG content and the expression of proteins involved in autophagy, were not increased by the addition of resveratrol. These observations are not in accordance with those reported by Dutta *et al.* [34], who found that a 20% energy restriction was not effective in inducing autophagy, and that the latter was only activated when the energy restricted diet was accompanied with resveratrol administration. The apparent discrepancy between our results and those reported by Dutta *et al.* again could be due to some differences in the experimental designs of the studies: the length of the experimental period (6 weeks *vs* 12 weeks), the age of the animals (6 weeks *vs* 25 months at the beginning of the experiment) and the selected resveratrol doses (30 mg/kg body weight/d *vs* 50 mg/kg body weight/d).

To determine the oxidative status, in the present study several parameters commonly studied for this purpose were measured [46-48]. It has been reported that resveratrol has anti-oxidant effects due to its scavenging activity on a variety of oxidants and the inhibition of reactive oxygen species (ROS) production [49]. In the present study, differences were only observed in TBARS formation, a parameter widely used as a marker of lipid peroxidation and oxidative damage. The three treated groups showed reduced levels of this parameter when compared with the C group. Regarding the other analysed oxidative markers, the lack of changes among the C group and the treated groups could be due to the fact that a high oxidative stress was not induced after 6 weeks of high-fat high-sucrose feeding. In fact, in previous studies from our group 12 weeks of high-fat high-sucrose feeding, but not 6 weeks (like in the present study) led to significantly increased MDA values when compared with control rats (data not shown). In addition, in the present experimental design, 6 weeks of standard feeding, after steatosis induction, could ameliorate the oxidative status. Consequently, we may

propose that higher oxidative stressing conditions could be needed for resveratrol to clearly show its anti-oxidative effect, and also that switching to standard feeding for 6 weeks might have masked the anti-oxidant effects of resveratrol.

In conclusion, the present data demonstrate that resveratrol treatment can partially reverse hepatic lipid accumulation previously induced by a high-fat high-sucrose diet, although less efficiently than mild energy restriction. Under our experimental conditions, the delipidating effect of energy restriction is mediated in part by the activation of autophagy; however, the involvement of autophagy in the effects of resveratrol is less clear. Taking into account our data and those reported in the literature, it can be suggested that resveratrol induced autophagy activation plays a less important role in hepatic steatosis treatment than it does in the prevention. Nevertheless, further studies are needed.

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Conflict of interest

The authors declare that they have no competing interests.

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Figures and tables

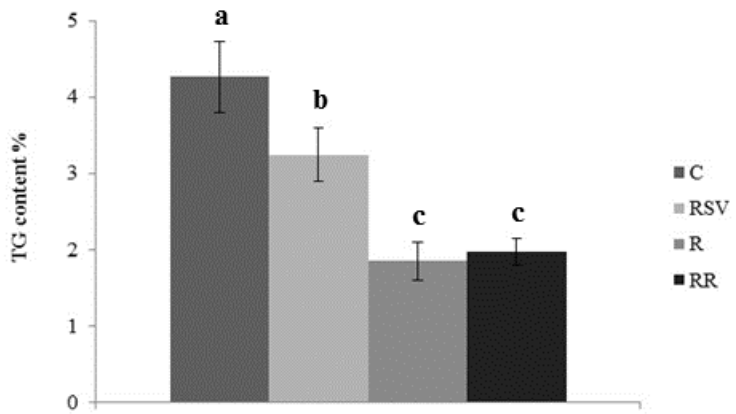

Figure 1. TG content, expressed as percentage, in liver from rats fed an obesogenic diet for 6 weeks and then fed a standard diet (C), or standard diet supplemented with resveratrol (RSV), or submitted to energy restriction and fed a standard diet (R), or submitted to energy restriction and fed a standard diet supplemented with resveratrol (RR) (n = 9/group) for additional 6 weeks. Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls post-hoc test. Values not sharing a common letter are significantly different ($p < 0.05$). TG: triacylglycerol.

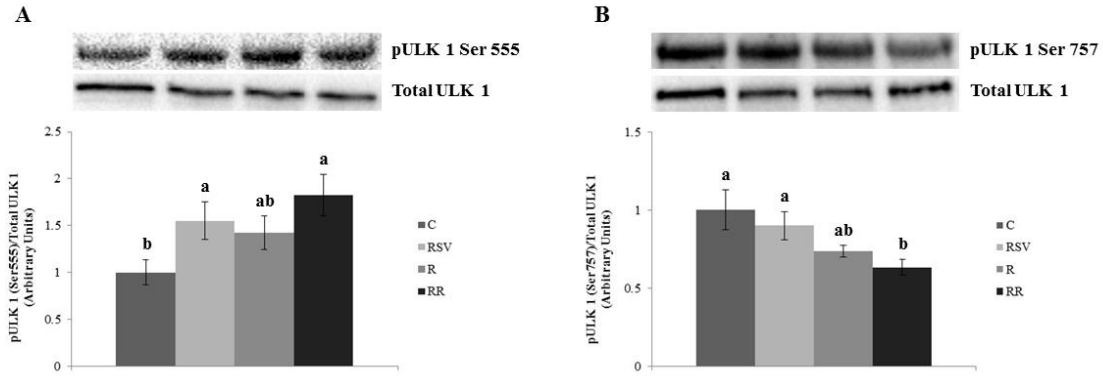

Figure 2. Phosphorylated ULK1 (Serine 555)/Total ULK1 (A) and phosphorylated ULK1 (Serine 757)/Total ULK1 (B) ratios in livers from rats fed an obesogenic diet for 6 weeks and then fed a standard diet (C), or standard diet supplemented with resveratrol (RSV), or submitted to energy restriction and fed a standard diet (R), or submitted to energy restriction and fed a standard diet supplemented with resveratrol (RR) (n =

9/group) for additional 6 weeks. Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls post-hoc test. Values not sharing a common letter are significantly different ($p < 0.05$). ULK1: UNC-51-like kinase-1.

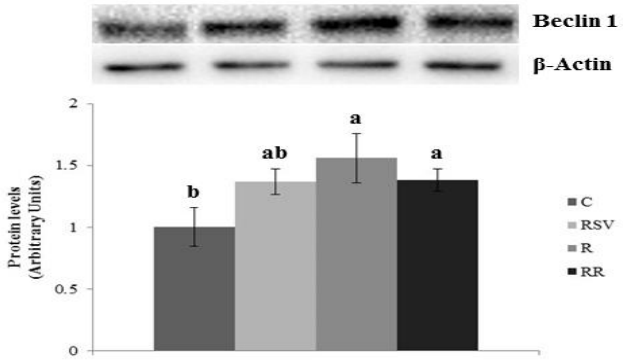

Figure 3. Beclin 1 protein expression in livers from rats fed an obesogenic diet for 6 weeks and then fed a standard diet (C), or standard diet supplemented with resveratrol (RSV), or submitted to energy restriction and fed a standard diet (R), or submitted to energy restriction and fed a standard diet supplemented with resveratrol (RR) (n = 9/group) for additional 6 weeks. Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls post-hoc test. Values not sharing a common letter are significantly different ($p < 0.05$). Beclin 1: Mammalian homolog of yeast Atg 6.

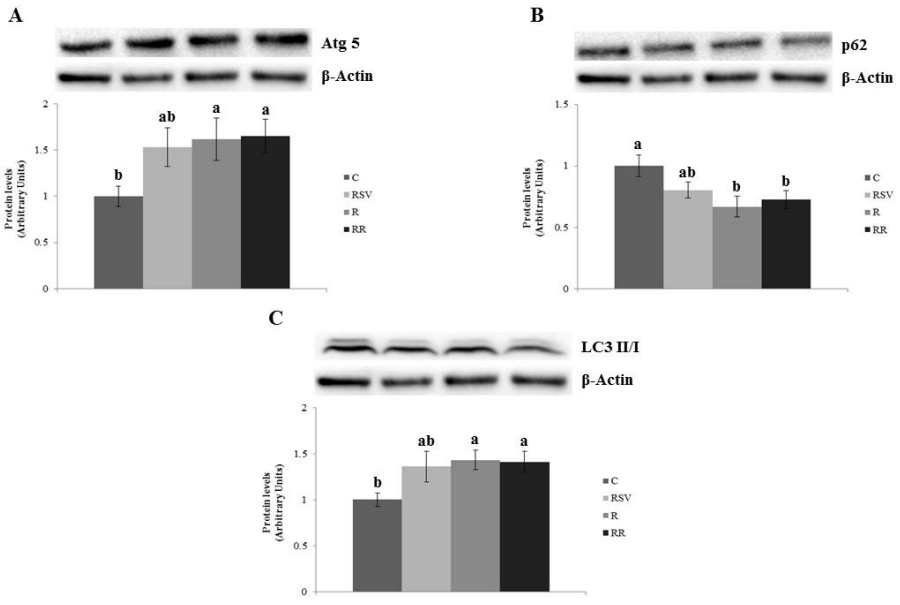

Figure 4. Atg 5 (A) and p62 (B) protein expressions, and LC3 II/I (C) expression ratio in livers from rats fed an obesogenic diet for 6 weeks and then fed a standard diet (C), or standard diet supplemented with resveratrol (RSV), or submitted to energy restriction and fed a standard diet (R), or submitted to energy restriction and fed a standard diet supplemented with resveratrol (RR) (n = 9/group) for additional 6 weeks. Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls post-hoc test. Values not sharing a common letter are significantly different ($p < 0.05$). Atg 5: Autophagy protein 5.

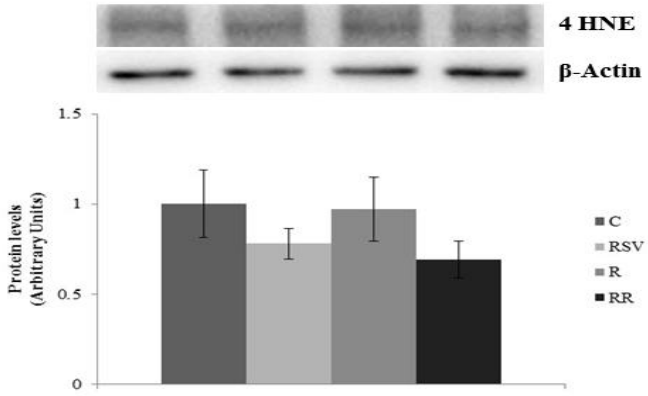

Figure 5. 4 HNE protein expression in livers from rats fed an obesogenic diet for 6 weeks and then fed a standard diet (C), or standard diet supplemented with resveratrol (RSV), or submitted to energy restriction and fed a standard diet (R), or submitted to energy restriction and fed a standard diet supplemented with resveratrol (RR) (n = 9/group) for additional 6 weeks. Values are means ± SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls post-hoc test. Values not sharing a common letter are significantly different ($p < 0.05$). 4 HNE: 4 Hydroxynonenal.

Table 1. Oxidative stress parameters in liver of rats fed on the experimental diets for 6 weeks.

	C	RSV	R	RR	ANOVA
TBARS (µM MDA/mg protein)	1.70 ± 0.08 [a]	1.46 ± 0.05 [b]	1.29 ± 0.07 [bc]	1.27 ± 0.05 [c]	$p < 0.05$
Total glutathione (µM/mg protein)	12.7 ± 2.6	8.8 ± 0.6	12.2 ± 2.2	15.1 ± 3.8	NS
GSH:GSSG ratio	14.5 ± 2.8	10.9 ± 1.2	14.4 ± 4.0	11.8 ± 2.2	NS
GPx (mU/mg protein)	31.9 ± 3.3	37.2 ± 2.2	39.8 ± 3.0	38.8 ± 2.4	NS
SOD (% inhibition rate/mg protein)	23.0 ± 4.0	24.6 ± 5.7	19.9 ± 5.6	16.2 ± 2.2	NS

TBARS, thiobarbituric acid reactive species; MDA, malonaldehyde; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase.

Values are mean±SEM. Differences among groups were determined by using one-way ANOVA followed by Newman Keuls *post-hoc* test. Values not sharing a common letter are significantly different. NS: Not significant.