Resveratrol Modulates Bovine Adipocyte Apoptosis by Activating the SIRT1/AMPK Signaling Pathway Postprint

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Abstract

This study aimed to investigate the effects of the plant extract resveratrol (RES) on the apoptosis rate of bovine subcutaneous adipocytes and the mRNA and protein expression levels of key factors in the sirtuin 1 (SIRT1)/adenosine monophosphate-activated protein kinase (AMPK) signaling pathway. Subcutaneous preadipocytes from 18-month-old Luxi Yellow cattle were selected and, on day 0 of cell differentiation, the culture medium was replaced with media containing RES at concentrations of 0 (control), 100, 200, and 400 mol/L for 48 h of treatment, with three replicates per group. Hoechst 33342 staining was used to detect morphological changes in cell apoptosis, flow cytometry was used to detect the apoptosis rate, quantitative real-time PCR (qPCR) and Western blotting were used to detect the mRNA and protein expression levels of key factors in the SIRT1/AMPK signaling pathway including SIRT1, AMPKα, forkhead transcription factor 1 (FoxO1), B-cell lymphoma/leukemia-2 (Bcl-2), cysteine-aspartic protease-3 (caspase-3), and pro-apoptotic protein Bcl-2-associated X protein (Bax), and Oil Red O staining was used to identify adipocytes. The results showed that, compared with the control group: the apoptosis rate of bovine subcutaneous adipocytes treated with different concentrations of RES was extremely significantly increased ($P<0.01$); the mRNA expression levels of SIRT1, AMPKα, caspase-3, and Bax were significantly or extremely significantly increased ($P<0.05$ or $P<0.01$), while the mRNA expression level of Bcl-2 was extremely significantly decreased ($P<0.01$); the protein expression levels of SIRT1, AMPKα, and caspase-3 were significantly or extremely significantly increased ($P<0.05$ or $P<0.01$); after treatment with 200 and 400 mol/L RES, the mRNA and protein expression levels of FoxO1 were significantly or extremely significantly increased ($P<0.05$ or $P<0.01$), the protein expression level of Bax was extremely significantly increased ($P<0.01$), and the protein expression level of Bcl-2 was extremely significantly decreased ($P<0.01$). After treatment with

100 mol/L RES, the mRNA and protein expression levels of FoxO1 as well as the protein expression levels of Bcl-2 and Bax showed no significant difference from the control group (P>0.05). These results indicate that RES promotes apoptosis of bovine subcutaneous adipocytes by activating the SIRT1/AMPK signaling pathway and concurrently activating the downstream factor FoxO1, providing a theoretical basis for reducing bovine subcutaneous fat deposition through nutritional regulation technology.

Full Text

Resveratrol Induces Apoptosis of Bovine Adipocytes through Activation of Sirtuin Type 1/Adenosine Monophosphate Activated Protein Kinase Signaling Pathway

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Abstract: This study investigated the effects of the plant extract resveratrol (RES) on apoptosis rate and mRNA and protein expression of key factors in the sirtuin type 1 (SIRT1)/adenosine monophosphate activated protein kinase (AMPK) signaling pathway in bovine subcutaneous adipocytes. Subcutaneous preadipocytes from 18-month-old Luxi yellow cattle were cultured and, on day 0 of differentiation, treated for 48 h with culture medium containing 0 (control), 100, 200, or 400 mol/L RES, with three replicates per group. Hoechst 33342 staining was used to detect morphological changes in apoptotic cells, flow cytometry was employed to measure apoptosis rate, and quantitative real-time PCR (qPCR) and Western blot were used to assess mRNA and protein expression of key SIRT1/AMPK signaling pathway factors including SIRT1, AMPKα, forkhead box protein O1 (FoxO1), B cell lymphoma/leukemia-2 (Bcl-2), cysteine-aspartate protease-3 (caspase-3), and Bcl-2 associated X protein (Bax). Adipocytes were identified by Oil Red O staining. The results showed that compared with the control group, RES treatment at all concentrations significantly increased adipocyte apoptosis rate (P<0.01). RES treatment also significantly or extremely significantly increased mRNA expression of SIRT1, AMPKα, caspase-3, and Bax (P<0.05 or P<0.01), while extremely significantly decreasing Bcl-2 mRNA expression (P<0.01). Protein expression of SIRT1, AMPKα, and caspase-3 was significantly or extremely significantly increased by RES treatment (P<0.05 or P<0.01). At RES concentrations of 200 and 400 mol/L, FoxO1 mRNA and protein expression and Bax protein expression were significantly or extremely significantly increased (P<0.05 or P<0.01), while Bcl-2 protein expression was extremely significantly decreased (P<0.01). Treatment

with 100 mol/L RES did not significantly affect FoxO1 mRNA and protein expression or Bcl-2 and Bax protein expression compared with the control group (P>0.05). These findings demonstrate that RES promotes apoptosis of bovine subcutaneous adipocytes by activating the SIRT1/AMPK signaling pathway and its downstream target FoxO1, providing a theoretical basis for reducing subcutaneous fat deposition in cattle through nutritional regulation.

Keywords: SIRT1; AMPK; FoxO1; resveratrol; bovine; adipocytes; apoptosis

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Adipocyte apoptosis represents the programmed death of energy storage cells. Regulation of adipocyte number through apoptosis is considered essential for maintaining normal physiological function during adipose tissue growth and differentiation, working in concert with proliferation and differentiation to maintain constant cell numbers throughout the body. In-depth investigation of adipocyte apoptosis and its regulatory mechanisms holds significant research value for treating human obesity-related diseases and controlling animal fat deposition to improve meat quality. The link between fat reduction, decreased adipocyte number, and adipocyte apoptosis was only established in the 1990s following the discovery of apoptosis in human adipocytes. Subsequent research has focused primarily on the 3T3-L1 adipocyte cell line, as well as primary cultured adipocytes from humans, rodents, and porcine preadipocytes. Our research team previously investigated differences in apoptosis between subcutaneous and intramuscular preadipocytes from Luxi yellow cattle, finding that sirtuin type 1 (SIRT1) regulates apoptosis in bovine preadipocytes through forkhead box protein O1 (FoxO1) and its target genes, with higher SIRT1 expression and apoptosis rates in subcutaneous compared with intramuscular adipocytes. However, the underlying regulatory mechanisms require further investigation.

SIRT1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase belonging to the silent information regulator 2 (Sir2) family. It is closely associated with cell proliferation, differentiation, aging, apoptosis, and metabolism, making it a hot topic in life sciences research. Adenosine monophosphate activated protein kinase (AMPK) binds to adenosine monophosphate (AMP) and regulates enzyme activity by sensing cellular energy levels and metabolic balance, earning it the designation of an "energy switch" that participates in regulating cell proliferation and apoptosis. As an energy sensor and regulator, AMPK serves as an integration hub for numerous signaling pathways and shares intrinsic connections with SIRT1, together playing crucial regulatory roles in energy metabolism. The complex relationship between AMPK and SIRT1 and their interactions with upstream and downstream signaling molecules in adipocyte apoptosis warrant further investigation.

Resveratrol (RES), a non-flavonoid polyphenolic compound derived from plants such as peanuts, grapes (red wine), polygonum cuspidatum, and mulberries, exhibits multiple biological and pharmacological activities including antioxidant,

neuroprotective, cardioprotective, antitumor, and anti-aging effects. RES is commonly used as a SIRT1 activator and has been extensively studied in humans and model animals, particularly in epigenetic regulation where it has achieved remarkable results. However, as a plant extract, research in animals, especially ruminants, has focused primarily on phenotypic aspects with few mechanistic studies. For example, studies in sheep have examined RES supplementation for reducing methane emissions, while feeding grape marc to dairy cows altered rumen bacterial and archaeal communities and decreased methane production by approximately 20%. Significant gaps remain in our understanding of RES effects on animal growth, nutrient digestion, and epigenetic regulatory mechanisms, necessitating systematic and detailed investigation. Therefore, this study examined RES effects on apoptosis rate and mRNA and protein expression of key genes in the SIRT1/AMPK signaling pathway in bovine subcutaneous adipocytes, aiming to provide a theoretical foundation for reducing subcutaneous fat deposition in beef cattle through nutritional regulation.

Materials and Methods

Experimental Animals

Three healthy, disease-free 18-month-old Luxi yellow cattle steers weighing approximately 450 kg were selected. After slaughter, subcutaneous adipose tissue was aseptically isolated. The experiment was approved by the Animal Care and Use Committee of Shandong Academy of Agricultural Sciences (IACUC20060101) and complied with relevant regulations and institutional rules for experimental animal welfare.

Cell Culture and Treatment

Bovine Subcutaneous Adipocyte Culture Subcutaneous adipose tissue was minced into 1 mm^3 pieces and digested with collagenase I solution [Dulbecco' s modified Eagle medium/nutrient mixture F12 (DMEM/F12) + 1 g/L collagenase I] in a shaking water bath at 37°C for 30 min. Growth medium [DMEM/F12 + 10% fetal bovine serum (FBS)] was added to terminate digestion, and the mixture was filtered through a 200-mesh cell strainer. The filtrate was centrifuged at 1,385×g for 5 min using a low-speed centrifuge (TDL-40B, Shanghai Anting), the supernatant was discarded, and the pellet was washed twice with DMEM/F12 (692.5×g, 10 min each). Cells were resuspended in growth medium, counted, and plated to obtain bovine preadipocytes. After cells reached confluence, the DMEM/F12 growth medium was replaced with induction differentiation medium [DMEM/F12 + 10% FBS + 1% penicillin-streptomycin stock solution + 5 g/mL insulin (INS) + 1 mol/L dexamethasone (DEX) + 0.5 mmol/L 1-methyl-3-isobutylmethylxanthine (IBMX)] to induce preadipocyte differentiation. After 48 h, the induction medium was replaced with basal differentiation medium (DMEM/F12 + 10% FBS + 1% penicillin-streptomycin stock solution + 5 g/mL INS), which was changed every 2 days thereafter. Generally, by day 8, over 80% of preadipocytes had differentiated

into mature adipocytes. RES, collagenase I, FBS, DMEM/F12, and penicillin-streptomycin stock solution were purchased from Gibco (USA); INS, DEX, and IBMX were purchased from Sigma (USA).

Hoechst 33342 Staining Cell climbing slides were prepared. On day 0 of differentiation, the medium was replaced with culture medium containing 0 (control), 100, 200, or 400 mol/L RES, with three replicates per group. After 48 h of treatment, the medium was removed and cells were washed three times with phosphate buffer saline (PBS). Staining was performed according to the Hoechst 33342 staining kit instructions (Beyotime Biotechnology, Shanghai), and images were captured using an Olympus Microscope Digital Camera Model DP71 imaging system and Olympus BX51 fluorescence microscope.

Oil Red O Staining Cell climbing slides were prepared. On day 8 of differentiation, the medium was replaced with culture medium containing 0 (control), 100, 200, or 400 mol/L RES, with three replicates per group. After 48 h, adipocytes were identified by Oil Red O staining (Nanjing Jiancheng Bioengineering Institute): the medium was removed, cells were washed three times with PBS, fixed with 10% formaldehyde in isotonic salt buffer for 40 min, rinsed with PBS, stained with 10 mL Oil Red O working solution for 30 min, differentiated with 60% isopropanol for 10-20 s, rinsed with tap water, counterstained with hematoxylin for 10 min, rinsed again, mounted with glycerin gelatin, and photographed.

Flow Cytometry Analysis of Adipocyte Apoptosis Cells were seeded at 5$×10^{4}$ cells/cm^2 in 25 cm^2 culture flasks. On day 0 of subcutaneous preadipocyte differentiation, the medium was replaced with culture medium containing 0 (control), 100, 200, or 400 mol/L RES, with three replicates per group. After 48 h, cells were collected from the flasks and apoptosis was detected using annexin V fluorescein isothiocyanate (Annexin V FITC)/propidium iodide (PI) double staining according to the Annexin V-FITC/PI apoptosis detection kit instructions (KeyGen Biotech, Jiangsu). Three control samples were prepared to set fluorescence compensation and quadrant gates on the BD FACSCalibur flow cytometer: (a) unstained cells, (b) cells stained only with Annexin V FITC, and (c) cells stained only with PI. Results were analyzed using the BD FACSCalibur platform, with apoptosis rate determined by counting cells in the apoptotic region.

Quantitative Real-Time PCR (qPCR) Cells were seeded at 5$×10^{4}$ cells/cm^2 in 25 cm^2 culture flasks. On day 0 of differentiation, the medium was replaced with culture medium containing 0 (control), 100, 200, or 400 mol/L RES, with three replicates per group. After 48 h of intervention, total cellular RNA was extracted using the RNApure ultra-pure total RNA rapid extraction kit (Aidlab Biotechnologies, Beijing). RNA quality and purity were assessed by 1% agarose gel electrophoresis and UV spectrophotometry. Two-step qPCR was

performed using the Transcriptor™ First Strand cDNA Synthesis Kit (Roche). After reverse transcription, qPCR was conducted using the ChamQ™ SYBR® Color qPCR Master Mix (Vazyme Biotech, Nanjing) with 1 L cDNA template in 96-well plates on a Roche 480 real-time PCR system. The reaction protocol consisted of pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s, with a melting curve analysis (95°C for 15 s, 60°C for 60 s, 95°C for 15 s). Primers for SIRT1 and related genes were designed and synthesized by Shanghai BioSune Biotechnology (primer sequences shown in Table 1). β-actin served as the internal reference, and relative expression levels were calculated using the 2^(-ΔΔCt) method, where Δt = Ct(target gene) - Ct(reference gene), ΔΔt = Δt(treatment group) - Δt(control group), with the control group expression level set as 1.

Western Blot Analysis Cells were seeded at 5$×10^{4}$ cells/cm^2 in 25 cm^2 culture flasks. On day 0 of differentiation, the medium was replaced with culture medium containing 0 (control), 100, 200, or 400 mol/L RES, with three replicates per group. After 48 h, total cellular protein was extracted and quantified. Thirty micrograms of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN Tetra Cell system (Bio-Rad, USA), then transferred to polyvinylidene fluoride (PVDF) membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were incubated with primary antibodies overnight at 4°C, washed three times with Tris-buffered saline with Tween (TBST) for 10 min each, incubated with secondary antibodies for 2 h, washed again, and detected with chemiluminescent substrate. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the loading control. Protein bands were visualized using a Gel Doc XR+ gel imaging system (Bio-Rad) and analyzed with Quantity One software to determine band density and expression levels. Target protein expression was normalized to GAPDH density, with relative expression presented as the ratio of each group' s gray value to the control group' s gray value. Antibodies against SIRT1, Bax, and GAPDH were from Abcam (UK); caspase-3 and Bcl-2 were from Cell Signaling Technology (CST, USA); FoxO1 was from Novus Biologicals (USA); and AMPKα was from GeneTex (USA).

Statistical Analysis

Data are presented as mean ± standard error (SE). One-way ANOVA was performed using SPSS 21.0 software, with pairwise comparisons conducted using Tukey' s test. $P<0.05$ was considered significant, $P<0.01$ extremely significant, and $P>0.05$ not significant.

Results

Identification of Bovine Subcutaneous Adipocytes

Oil Red O staining is a specific method for identifying adipocytes, as intracellular lipid droplets are stained red by the lipophilic dye while other cells lacking lipid droplets remain unstained, thereby confirming the adipocyte phenotype. As shown in Figure 1 [Figure 1: see original paper], both control and RES groups stained positive with Oil Red O, confirming that the cultured cells were adipocytes. Oil Red O staining intensity decreased with increasing RES concentration.

Effects of Resveratrol on Bovine Subcutaneous Adipocyte Apoptosis

As shown in Figure 2 [Figure 2: see original paper]-A and 2-B, flow cytometry analysis and apoptosis rate measurements revealed that treatment of bovine subcutaneous adipocytes with different RES concentrations on day 0 of differentiation for 48 h resulted in extremely significantly higher apoptosis rates compared with the control group ($P<0.01$). Hoechst 33342, which permeates cell membranes and emits strong blue fluorescence upon DNA binding, is commonly used for apoptosis detection. As shown in Figure 2-C, compared with the control group, RES treatment for 48 h resulted in condensed and intensely stained nuclei in apoptotic cells under fluorescence microscopy. At 400 mol/L RES, fewer cells were observed due to extensive apoptosis and cell detachment, but remaining cells exhibited fragmented, densely stained nuclei.

Effects of RES on mRNA and Protein Expression of SIRT1, AMPKα, and FoxO1

As shown in Table 3 and Figure 3 [Figure 3: see original paper], compared with the control group, SIRT1 mRNA expression increased significantly or extremely significantly with increasing RES concentration ($P<0.05$ or $P<0.01$), and protein expression also increased extremely significantly ($P<0.01$). AMPKα mRNA and protein expression increased significantly or extremely significantly ($P<0.05$ or $P<0.01$). At RES concentrations of 200 and 400 mol/L, FoxO1 mRNA and protein expression increased significantly or extremely significantly ($P<0.05$ or $P<0.01$). Treatment with 100 mol/L RES did not significantly affect FoxO1 mRNA or protein expression compared with the control group ($P>0.05$). Both gene and protein expression generally showed concentration-dependent increases.

Effects of RES on mRNA and Protein Expression of Caspase-3, Bax, and Bcl-2

As shown in Table 3 and Figure 4 [Figure 4: see original paper], compared with the control group, Bcl-2 mRNA expression decreased extremely significantly with increasing RES concentration ($P<0.01$), and Bcl-2 protein expression also decreased extremely significantly at 200 and 400 mol/L RES ($P<0.01$).

Caspase-3 mRNA and protein expression increased significantly or extremely significantly ($P<0.05$ or $P<0.01$). Bax mRNA expression increased significantly or extremely significantly ($P<0.05$ or $P<0.01$), and Bax protein expression increased extremely significantly at 200 and 400 mol/L RES ($P<0.01$). Treatment with 100 mol/L RES did not significantly affect Bcl-2 or Bax protein expression compared with the control group ($P>0.05$).

Discussion

Resveratrol and Adipocyte Apoptosis

Research on adipose tissue apoptosis began relatively late due to the presence of large lipid droplets in adipocytes that prevent cell shrinkage and apoptotic body formation, combined with the low density of adipocytes that causes them to float on liquid surfaces, making them difficult to wash and separate during manipulation. Apoptosis is a genetically controlled process of programmed cell death, with the Bcl-2 family serving as one of the most important regulatory factors. This family comprises two functional classes: pro-apoptotic members such as Bax and Bad that increase mitochondrial outer membrane permeability and release of cytochrome c, thereby activating inactive caspase zymogens and ultimately inducing apoptosis; and anti-apoptotic members such as Bcl-2 and Bcl-xl that stabilize mitochondrial membranes and prevent cytochrome c release, thus negatively regulating the mitochondrial apoptosis pathway.

Studies on 3T3-L1 preadipocytes treated with RES demonstrated increased SIRT1 protein expression and activation of caspase-3 and caspase-9. RES specifically increased SIRT1 expression and activity in porcine preadipocytes, with SIRT1 upregulation affecting caspase-3 and Bcl-2 family factor activities while participating in transcriptional regulation of tumor suppressor p53 and nuclear factor kappa B (NF- B), suggesting that SIRT1-mediated regulation of apoptosis-related factors is a key mechanism underlying RES-induced preadipocyte apoptosis. In the present study, RES specifically increased SIRT1 expression and activity in bovine adipocytes, elevated caspase-3 and Bax mRNA and protein expression, and decreased Bcl-2 mRNA and protein expression, indicating that pro-apoptotic factor activity was significantly enhanced while anti-apoptotic factor activity was diminished during RES-induced bovine adipocyte apoptosis, consistent with previous findings. In contrast, when rat H9c2 cells were exposed to hypoxic conditions for 24 h, apoptosis increased, but subsequent RES treatment for 24 h under the same conditions significantly reduced apoptotic cells. Treatment of human degenerative disc nucleus pulposus (NP) cells with RES for 48 h decreased apoptosis levels and caspase-3 activation, with SIRT1 primarily regulating NP cell survival through the protein kinase B (Akt) anti-apoptotic signaling pathway. These findings suggest that the functional regulation of apoptosis by RES-induced SIRT1 is highly dependent on cell type, developmental stage, environmental conditions, and other factors.

While RES has been extensively studied as a SIRT1 activator in humans and

model animals, research in animals, particularly ruminants, has focused mainly on methane emission reduction. This study provides a preliminary investigation of RES effects on bovine subcutaneous adipocyte apoptosis, highlighting the need for further systematic research on fat deposition regulation in beef cattle.

SIRT1/AMPK Signaling Pathway and Adipocyte Apoptosis

As a highly conserved protein kinase family in mammals, AMPK serves as a central component in protein kinase cascades and plays a pivotal role in signal transduction mechanisms regulating energy metabolism. SIRT1, as an NAD^+-dependent histone deacetylase, regulates cellular energy metabolic balance through changes in intracellular redox status. Studies have demonstrated intrinsic connections between AMPK and SIRT1 that play important regulatory roles in energy metabolism. Energy depletion or AMPK activation leads to SIRT1 activation, potentially through increased NAD^+ or NAD^+/NADH ratio and/or nicotinamide phosphoribosyl transferase (NAMPT), with SIRT1 deacetylating and activating liver kinase B1 (LKB1), which subsequently activates AMPK. RES can influence AMPK through SIRT1 activation. In studies of RES-induced apoptosis in murine 3T3-L1 preadipocytes, SIRT1 activation inhibited Akt activity while increasing AMPK activity, with the SIRT1/AMPK signaling pathway activating mitochondria-mediated signaling cascades. Additionally, SIRT1 acts on numerous transcription factors including FoxO1, myogenic determination gene (MyoD), and p53. RES upregulated SIRT1, FoxO1, and adiponectin gene expression in human visceral adipocytes. Similar to these findings, the present study showed that RES specifically increased SIRT1 expression and activity in bovine adipocytes, with corresponding increases in AMPKα and FoxO1 mRNA and protein expression, demonstrating that RES-induced bovine adipocyte apoptosis involves activation of the SIRT1/AMPK signaling pathway and its downstream target FoxO1, leading to enhanced pro-apoptotic effects. However, contradictory results exist, such as RES inhibiting the AMPK signaling pathway in human skeletal muscle cells. Other studies have shown that the AMPK $ $2 catalytic subunit isoform is significantly downregulated in hepatocellular carcinoma (HCC), with ectopic AMPK expression enhancing p53 acetylation and stability in liver cancer cells. p53 deacetylation enables phosphorylation of SIRT1 at threonine-344 by AMPK, leading to SIRT1 inactivation, which promotes p53 acetylation and apoptosis in liver cancer cells. These findings indicate that RES-induced SIRT1/AMPK signaling pathway regulation of apoptosis is complex, exhibiting both anti-apoptotic and pro-apoptotic effects depending on cell type, treatment specificity, stimulation duration and intensity, and other factors.

Due to species-specific antibody limitations, this study only examined total protein levels. Future work will investigate phosphorylation levels of SIRT1/AMPK signaling pathway protein kinases and their subunits to elucidate the specific mechanisms of RES-induced AMPK pathway activation. Additionally, further research is needed on RES effects on bovine intramuscular adipocytes and mus-

cle cells, as well as large-scale feeding trials, to provide new insights for reducing subcutaneous fat deposition in beef cattle through nutritional regulation with plant extracts.

In conclusion, RES promotes apoptosis of bovine subcutaneous adipocytes by activating the SIRT1/AMPK signaling pathway and its downstream target FoxO1.

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Note: Figure translations are in progress. See original paper for figures.