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Modulating effects of steviol and steviol glycosides on adipogenesis, lipogenesis, glucose uptake and insulin resistance in 3T3-L1 adipocyte model

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ABSTRACT

Stevia rebaudiana Bertoni is famous for sweetness due to the content of steviol glycosides (SG). This study aimed to determine the effect of SG (stevioside, rebaudioside A) and steviol on adipogenesis and lipogenesis in the 3T3-L1 model. Test compounds were also analysed for their ability to affect glucose uptake in hypertrophied insulin-resistant 3T3-L1 adipocytes. The most active compound in modulating adipogenesis, lipogenesis and insulin resistance was steviol, SG metabolism product. Steviol (10 μM, 100 μM) significantly down-regulated the expression of adipogenic transcription factors (PPARγ, C/EBPα, SREBP1) and lipogenic genes (FAS, aP2, LPL), which caused decreased lipid accumulation and triglyceride content in adipocytes. Treatment of insulin-resistant adipocytes with steviol (1 μM) and stevioside (1 μM, 10 μM) increased GLUT-4 transcript level and improved glucose uptake. Steviol also lowered resistin gene expression, which may mitigate insulin resistance in hypertrophied adipocytes. This study promotes SG as potential health-promoting ingredients in low-calorie functional foods.

1. Introduction

Excessive accumulation of adipose tissue can negatively affect health, leading to the development of obesity with all the consequences associated with it (World Health Organization. Obesity and overweight, 2021, Available online: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed on 15 November 2021), n.d.). Despite the increasing awareness about health, nutrition, physical activity and metabolic diseases, the problem of obesity is still a growing concern. It has been found that overweight currently leads to fatal consequences more often than underweight (Centers for Disease Control and Prevention. Adult Obesity Facts. 2020, Available online: https://www.cdc.gov/obesity/data/adult.html (accessed on 17 November 2021), n.d.). Excess body weight is a risk factor for many severe health conditions, such as stroke, heart disease or type 2 diabetes, and predisposes to developing certain types of cancer. That is why it is crucial to conduct research discovering new, safe and effective methods of preventing and treating obesity and diseases associated with excess body weight. More and more reports show the potential of natural substances that can support the treatment of obesity (Gamboa-Gómez et al., 2015, pp. 2015–2186; Ríos et al., 2015). It is known that plant bioactive compounds can interfere with the mechanisms regulating hypertrophic and hyperplastic growth of adipose tissue. Hypertrophy (expansion of existing adipocytes) is associated with insulin resistance and other metabolic disturbances (Jo et al., 2009). Abnormal excessive adipocyte expansion is accompanied by increased production of pro-inflammatory mediators, adipokine secretion dysregulation, dysfunctional fatty acid accumulation and metabolism, and disturbances in the insulin signal

Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; aP2, adipocyte protein 2; C/EBPα, CCAAT/enhancer-binding protein α; DMM, differentiation maintenance medium; DM, differentiation medium; DMEM, Dulbecco's Modified Eagle's Medium; FAS, fatty acid synthase; FBS, fetal bovine serum; GLUT-4, glucose transporter type 4; KRPH, Krebs Ringer phosphate HEPES; LPL, lipoprotein lipase; PPARγ, peroxisome proliferator-activated receptor gamma; RA, rebaudioside A; RGZ, rosiglitazone; SREBP1, sterol regulatory element-binding protein 1; STL, steviol; SG, steviol glycosides; ST, stevioside; TG, triglycerides.

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transduction pathway that promote insulin resistance (Schuster, 2010). Natural bio-actives have also been found to influence adipose tissue growth by inhibiting adipocyte differentiation, reducing lipogenesis, increasing lipolysis, and alleviating obesity-related chronic low-grade inflammation and insulin resistance.

Stevia rebaudiana Bertoni may be indicated as a promising candidate with potential anti-obesity effects and the capacity to mitigate concomitants linked to obesity. *Stevia rebaudiana* Bertoni is a plant that has been used for hundreds of years in traditional medicine in South America (Mehta et al., 2011). It is a small perennial with leaves that are used for calorie-free sweetening of food in many countries. The bioactive properties of *Stevia rebaudiana* have been intensively investigated in recent years. Several experimental studies have shown that stevia-derived compounds may have a broad spectrum of health-promoting properties, including anti-inflammatory, antihyperglycaemic, anti-parasitic, antioxidant and antiviral potential (U. Ahmad & Ahmad, 2018; Borgo et al., 2021). Recent studies have also reported that stevia-derived compounds and extracts may have a therapeutic effect in disorders associated with excess body weight and dyslipidaemia (Carrera-Lanestosa et al., 2017; Farhat et al., 2019; Kurek et al., 2020). It is believed that some of the health-promoting properties of stevia are attributable to steviol glycosides (SG), compounds responsible for the sweet taste of the leaves of the plant (J. Ahmad et al., 2020). Stevioside (ST) (Fig. 1A) and rebaudioside A (RA) (Fig. 1B) are diterpenes, the most abundant of all SG in the plant, and steviol (STL) (Fig. 1C) is their aglycon (Gerwig et al., 2016).

This study aimed to evaluate the effect of STL and SG on the processes of adipogenesis and lipogenesis and their potential to regulate the expression of genes responsible for carbohydrate and lipid metabolism in the 3T3-L1 adipocyte model under normal and insulin resistance conditions.

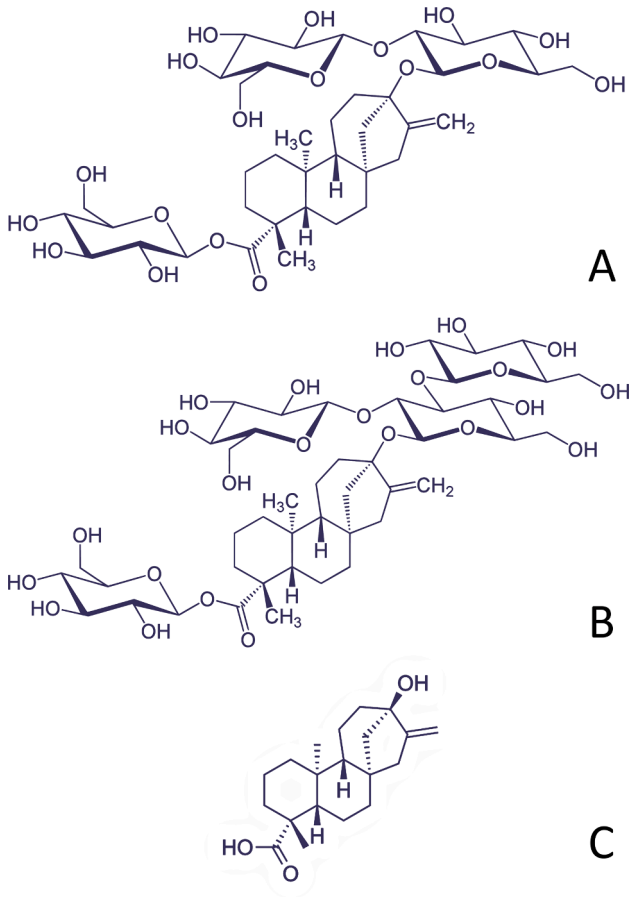

Fig. 1. Chemical structures of stevioside (**A**) rebaudioside A (**B**) and steviol (**C**).

2. Materials and methods

2.1. Test compounds

RA (≥98% HPLC) was obtained from Cayman Chemical, Michigan, USA and supplied by Biokom Baka Olszewski GP, Janki, Poland. ST (≥99%, 99.15% HPLC) and STL (≥98%, 99.46% HPLC) were obtained and supplied by Henan Allgreen Chemical Co., Ltd, Zhengzhou, China.

2.2. 3T3-L1 adipocytes, differentiation and experiment procedures

The mouse 3T3-L1 preadipocytes (ATCC, CL-173) were obtained from American Type Culture Collection (ATCC). They were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma–Aldrich, Poznań, Poland) supplemented with 10% (*v/v*) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific Polska, Warsaw, Poland) at 37 °C under a 5% CO2 atmosphere. For differentiation, the 3T3-L1 cells were seeded into 24-well plates at a density of 5×10^4 cells/well and grown until they reached confluence. After 2 days, cells were stimulated by a differentiation medium (DM) consisting of 0.25 μM dexamethasone (DEX) (Sigma–Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma–Aldrich), and 1 μM insulin (Sigma–Aldrich) in DMEM with 10% FBS. Cells were incubated in the DM for 48 h, and then the medium was replaced with a differentiation maintenance medium (DMM) containing DMEM, 10% FBS, and 1 μM insulin. After 48 h, DMM was replaced again with standard medium (DMEM/10% FBS).

The analysed compounds: RA, ST and STL, were suspended in DM, DMM, and standard medium and introduced into 3T3-L1 cell cultures at each differentiation stage. On day 8, adipocytes were collected for total RNA isolation and gene expression analysis using a real-time PCR method. In the medium, leptin and adiponectin protein levels were analysed using ELISA.

In another experiment, insulin resistance was induced in mature 3T3-L1 adipocytes differentiated according to the standard procedure by 10 ng/mL murine TNF-α (Sigma-Aldrich) for 5 days, with medium/TNF-α replacement every 2 days. Glucose uptake was measured in insulin-resistant adipocytes subjected to RA, ST and STL treatment.

2.3. Cell viability assay

The viability and metabolic activity of 3T3-L1 adipocytes treated with RA, ST, and STL were analysed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma–Aldrich, Poznań, Poland) following the previously described procedure (Olejnik et al., 2016).

2.4. Measurement of intracellular lipid content

The effect of RA, ST, and STL on lipid content in mature 3T3-L1 adipocytes was determined by the Oil Red O (Sigma–Aldrich, Poznań, Poland) staining method previously described (Kowalska et al., 2019) and by measuring total triglycerides (TG) using the Adipogenesis Assay Kit (Sigma–Aldrich, Poznań, Poland) following the manufacturer's instruction. Intracellular TG content was determined using an enzyme assay. A colourimetric product corresponding to the content of TG was measured at 570 nm (Tecan Infinite M200, Tecan Group Ltd., Männedorf, Switzerland). TG concentration was calculated based on the curve plotted for the TG standard. Intracellular TG content was normalized to cellular protein. The cell total protein concentration was determined using a BCA Protein Assay kit (Sigma-Aldrich, Poznań, Poland).

2.5. RNA extraction and gene expression analysis

TRI-Reagent (Sigma–Aldrich, Poznań, Poland) was used for total RNA isolation from 3T3-L1 adipocytes. Following the manufacturer's

instruction, the first-strand cDNA synthesis was performed with 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Polska, Warsaw, Poland). Gene expression level was quantified using a real-time PCR system (SmartCycler DX real-time PCR System Cepheid, Sunnyvale, CA, USA). PCR mixture in a final volume of 25 μL included 1 μL of cDNA sample, 5 μM/1 μL of specific forward and reverse primers, and 12.5 μL SYBR® Select Master Mix (Life Technologies, Carlsbad, CA, USA). Primer sequences used in this experiment are listed in Table 1. The real-time PCR reaction included an initial denaturation at 94 °C for 10 min, followed by 40 PCR cycles: 40 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C. The relative gene expression level in 3T3-L1 adipocytes was calculated using the $2^{-\Delta\Delta CT}$ method, and β-actin was used as an internal control/standard. The relative levels of gene transcripts were expressed as fold change compared with control (untreated) cells. All reactions were performed in triplicate.

2.6. Determination of adipokine production

Leptin and adiponectin secretion was measured with ELISA kits (Sigma–Aldrich, Poznań, Poland) following the manufacturer's protocols. Quantitation was performed using the calibration of standards.

2.7. Glucose uptake measurement in adipocytes

Glucose uptake assay was performed according to the modified method of Alonso-Castro and Salazar-Olivo (Alonso-Castro & Salazar-Olivo, 2008). Mature 3T3-L1 adipocytes were starved overnight in a serum-free medium (MEM containing BSA 0.5%). Subsequently, the medium was replaced with Krebs Ringer phosphate HEPES (KRPH) buffer containing 0.2% BSA (KRPH/BSA) and incubated for 60 min. The cells were then exposed for 60 min to RA, ST, and STL suspended in KRPH/BSA buffer supplemented with 80 μM 2-NBDG (2-N-7-(nitrobenz-2-oxa-1,3-diazol-4-yl)amino-2-deoxy-D-glucose) (Sigma-Aldrich) applied as fluorescent glucose analogue. The control culture was treated with 10 μM rosiglitazone (RGZ) (Sigma-Aldrich). After incubation, cultures were immediately washed with ice-cold PBS. The fluorescence intensity of 2-NBDG was measured at $\lambda_{ex} = 485$ nm and $\lambda_{em} = 535$ nm using a microplate reader Tecan M200 Infinite.

Table 1
The primer sequences used for real-time PCR analysis.

Gene	Accession	No. Sequence (5′ – 3′)	Amplicon (bp)
Mm *PPARγ*	NM-011146	F:TTTTCAAGGGTGCCAGTTTC R:AATCCTTGGCCCTCTGAGAT	198
Mm *C/EBPα*	NM-007678	F:TTACAACAGGCCAGGTTTCC R:GGCTGGCGACATACAGTACA	188
Mm *SREBP1*	NM-011480	F:TGTTGGCATCCTGCTATCTG R:AGGGAAAGCTTTGGGGTCTA	190
Mm *LEP*	NM-008493	F:GGATCAGGTTTTGTGGTGCT R:TTGTGGCCCATAAAGTCCTC	187
Mm*RESTN*	NM-022984.4	F: TCATTTCCCCTCCTTTTCCTTT R:TGGGACACAGTGGCATGCT	70
Mm *ADIPOQ*	NM-009605	F: CTGGCCACTTTCTCCTCATTTC R:GGCATGACTGGGCAGGATTA	120
Mm *aP2*	NM-024406	F:TCACCTGGAAGACAGCTCCT R: AATCCCCATTTACGCTGATG	182
Mm *LPL*	NM-008509	F:TCCAAGGAAGCCTTTGAGAA R:CCATCC TCAGTCCCAGAAAA	188
Mm *GLUT-4*	NM-001359114.1	F:TGCTGGGCACAGCTACCC R: CGGTCAGGCGCTTTAGAC	162
Mm *FAS*	NM-007988	F:TTGCTGGCACTACAGAATGC R:AACAGCCTCAGAGCGACAAT	192
Mm *ACTB*	NM-007393	F:CCACAGCTGAGAGGGAAATC R:AAGGAAGGCTGGAAAAGAG	193

2.8. Statistical analysis

Biological analyses were carried out in triplicate for each of the three experiments performed independently. Statistical analysis was performed using the STATISTICA version 13.3 software (Statsoft, Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to estimate the differences between the mean values of multiple groups. Levene's test verified the equality of variance assumption. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. The effect of steviol and steviol glycosides on cell viability, lipid accumulation and triglyceride content in 3T3-L1 adipocytes

In the first stage of the research, the influence of STL, ST and RA on the differentiation of the 3T3-L1 preadipocyte into mature fat cells was analysed. Adipocyte viability, lipid accumulation and intracellular TG content were determined to assess the effects of the compounds on preadipocyte differentiation, adipogenesis progression and overall condition of mature adipocytes. The results obtained indicate that the parameters analysed were not affected by RA or ST (Fig. 2A and 2B), regardless of the dose. In contrast, STL reduced lipid accumulation and TG content without significantly decreasing the viability of 3T3-L1 adipocytes (Fig. 2C). Namely, STL at a dose of 100 μM reduced intracellular lipid accumulation and TG content by 13.9% and 25.0%, respectively. Also, STL at the lower concentration (10 μM) decreased TG content by 19.5%. The microscopic image shows a reduction in lipid accumulation in adipocytes under the influence of STL at a dose of 100 μM compared to control non-treated adipocytes (Fig. 2D).

So far, the bioactivity of pure compound RA in the 3T3-L1 adipocyte cultures has not been reported in the literature. Studies performed in other experimental models (neonatal Sprague-Dawley rat cardiac fibroblasts, SH-SY5Y or HL-60 cell lines) also did not show any significant RA effect on cell proliferation (Prata et al., 2017; Rizzo et al., 2013). Research studies on the effects of ST and STL on 3T3-L1 adipocytes are scarce; however, those that are available in the literature confirm that the compounds do not affect adipocyte viability (Mohd-Radzman, 2013, p.; Noosa & Siripurkpong, 2016; Park et al., 2022). The influence of STL and SG on lipid accumulation and TG content in adipocytes has not been extensively investigated to date. Recently, Park et al. have first reported the effect of ST on adipogenesis in the 3T3-L1 adipocyte model. The intracellular lipids decreased dose-dependently after the treatment of differentiating 3T3-L1 cells with ST (Park et al., 2022). Anti-adipogenic and anti-lipogenic effects have been observed in 3T3-L1 preadipocytes treated with a differentiation medium supplemented with standardised formulations rich in RA and ST combined with green tea saponin E1 (RASE1). The inhibitory effect of RASE1 on lipogenesis was reflected in the reduced lipid accumulation and TG content (Khan et al., 2021). Another experiment with the α-TC1-6 cells exposed or non-exposed to palmitate showed that cell treatment with ST did not alter the intracellular TG content (Hong et al., 2006). *In vivo* studies provide data confirming that stevia-derived compounds can advantageously reduce TG content and significantly affect lipid metabolism (Kurek et al., 2020). Under physiological conditions, ST and RA are first degraded by intestinal microbiota to the aglycone and then absorbed as STL in the large bowel (Koyama et al., 2003; Wingard et al., 1980). Therefore, STL as the main metabolism product is probably responsible for the beneficial systemic effects of the oral administration of SG.

3.2. The effect of steviol and steviol glycosides on mRNA expression of adipogenic transcription factors in 3T3-L1 adipocytes

Adipogenesis-related processes strongly depend on transcription factors that interact synergistically, induce each other's expression, and cooperate to activate the expression of many adipogenic and lipogenic

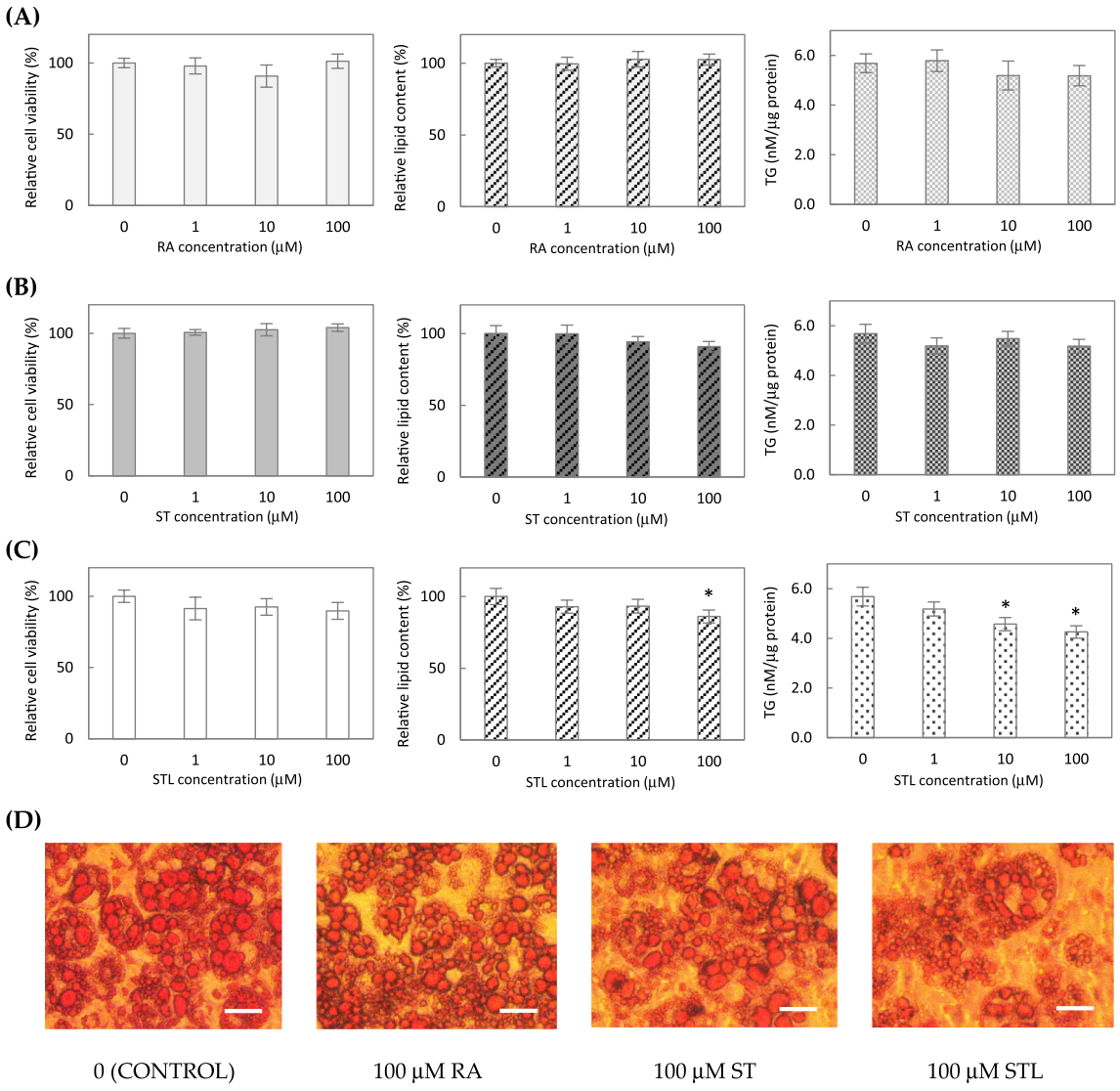

Fig. 2. Cell viability, lipid accumulation and TG content in 3T3-L1 adipocytes that underwent differentiation process with simultaneous treatment with rebaudioside (RA) (**A**), stevioside (ST) (**B**) and steviol (STL) (**C**). Microscopic images (**D**) present Oil Red O stained mature adipocytes treated with 100 µM RA, 100 µM ST and 100 µM STL. Scale bars indicate 50 µm. Differentiated 3T3-L1 cells stimulated by adipocyte differentiation medium without RA, ST, and STL treatment was the control (0). Significant differences at $^*p < 0.05$ when compared with control (Tukey's post hoc test).

genes (Guru et al., 2021). Thus, the transcription factors are crucial in adipocyte differentiation and proliferation, lipid metabolism, insulin sensitivity and apoptosis. Peroxisome proliferator-activated receptor gamma (PPARγ) is identified as a master transcriptional regulator in adipogenesis that determines the functionality of adipocytes. The important effect of PPARγ in adipocyte differentiation is activating the transcription factor CCAAT/enhancer-binding protein α (C/EBPα) (Ghaben & Scherer, 2019). Transcriptional cooperation between PPARγ and C/EBPα is essential to activating the programming of mature adipocytes. PPARγ can also react with molecules enhancing insulin sensitivity, which serve as its agonists and ligands. Since PPARγ is considered a central regulator in lipid and glucose homeostasis in response to various nutritional conditions, it is promoted as a drug and nutrient target for treating hyperlipidemia and diabetes (Sun et al., 2021). Additionally, sterol regulatory element-binding protein 1 (SREBP1) is an essential factor in the development of adipose tissue, which mediates the induction of lipid biosynthesis in adipocytes by increasing the gene expression of the main lipogenic genes (Shimano & Sato, 2017).

This study investigated the effect of RA, ST and STL on mRNA expression of the critical adipogenic transcription factors, including PPARγ, C/EBPα and SREBP1 in 3T3-L1 adipocytes. RA at a dose of 100 µM significantly down-regulated the mRNA expression of PPARγ, C/EBPα and SREBP1 (Fig. 3A). ST did not affect the mRNA expression of PPARγ; however, it significantly down-regulated the mRNA expression of C/EBPα at doses of 10 µM and 100 µM as well as lowered SREBP1 expression, when applied at a concentration of 10 µM (Fig. 3B). STL affected the expression of adipogenic transcription factors most strongly. It down-regulated the mRNA expression of PPARγ and SREBP1 at doses of 10 µM and 100 µM. STL at all concentrations tested was effective in inhibiting C/EBPα mRNA expression (Fig. 3C).

Data reported in recent years have also shown suppressed expression of adipogenic transcription factors in 3T3-L1 adipocytes treated with ST and SG (Khan et al., 2021; Park et al., 2022). ST has been found to reduce lipid accumulation during 3T3-L1 cell differentiation through down-regulation of PPARγ, SREBP-1, and C/EBPα protein expression and activation of AMPK signaling (Park et al., 2022). Similarly, RA- and ST-rich formulation (RASE1) inhibited 3T3-L1 adipocyte differentiation by down-regulating adipogenesis- and lipogenesis-promoting genes (Khan et al., 2021). Moreover, ST contributed to a significant reduction of PPARγ, C/EBPα and SREBP-1c mRNA expression in murine

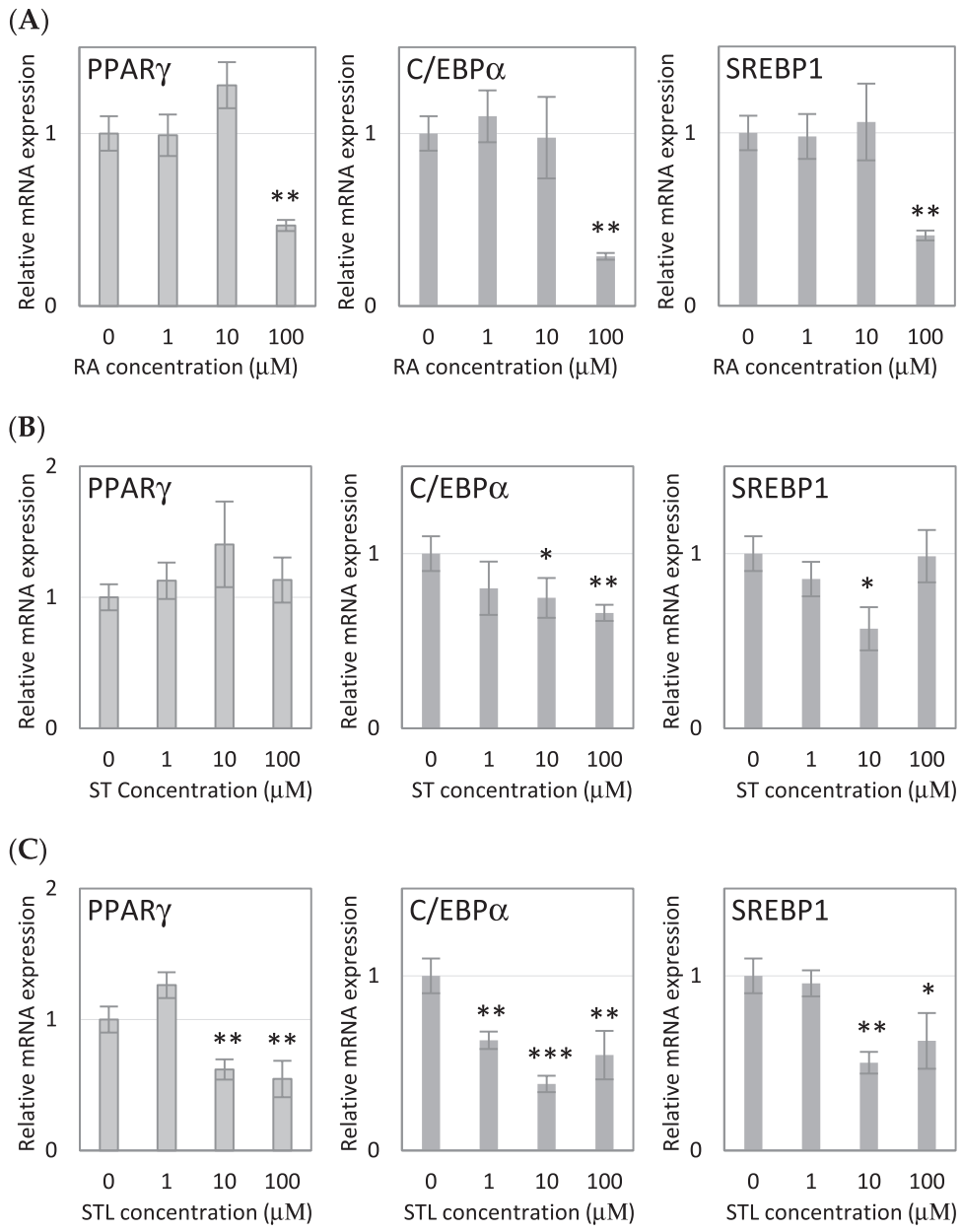

Fig. 3. Effect of RA (**A**), ST (**B**), and STL (**C**) on mRNA expression of adipogenic transcription factors PPARγ C/EBPα and SREBP1 in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells stimulated by adipocyte differentiation medium without RA, ST, and STL treatment was the control (0). Data are shown as relative gene expression (fold of control). Significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control (Tukey's post hoc test).

subcutaneous fat tissue (Kim et al., 2017) and the epididymal adipose tissue of db/db mice (Park et al., 2022). In contrast, treatment of α-TC1-6 cells with ST did not alter PPARγ and SREBP-1c expression in cultures without and with palmitate supplementation (Hong et al., 2006). Results obtained in the experiments suggest that stevia-derived compounds have the potential to inhibit the expression of transcription factors responsible for adipogenesis and could limit excessive intracellular lipid accumulation. However, despite interfering with the gene expression of adipogenic signaling molecules, RA and ST did not significantly affect the differentiation of pre-adipocytes into mature adipocytes. Contrary to RA and ST, significant molecular and cellular effects were observed in mature adipocytes treated with STL.

3.3. The effect of steviol and steviol glycosides on mRNA expression of genes involved in lipid and glucose metabolism in 3T3-L1 adipocytes

To examine the effects of RA, ST and STL on the regulation of lipid and glucose metabolism at the molecular level, the mRNA expression of the following genes: Fatty acid synthase (FAS), Adipocyte protein 2 (aP2), Lipoprotein lipase (LPL), Resistin (RESTN) and Glucose transporter type 4 (GLUT-4) was determined. The product of the FAS gene is an essential enzyme catalysing the synthesis of long-chain fatty acids *de novo* (Jiang et al., 2019). The aP2 gene encodes a protein whose function is to transport fatty acids. It binds to free fatty acids in the cytoplasm and modulates the inhibitory activity of released lipids. Also, it is a crucial factor in the maintenance of glucose homeostasis (Prentice et al., 2019). Lipoprotein lipase, encoded by the LPL gene, is responsible for the hydrolysis of triacylglycerols. It is essential in metabolic processes that determine the use and storage of fatty acids (Olivecrona, 2016). The role

of resistin - the product of the RESTN gene is still under discussion; it has been found that this protein participates in glucose metabolism regulation. Also, elevated resistin levels correlate with an exacerbation of insulin resistance and inflammation (Acquarone et al., 2019). GLUT-4 is one of the essential genes affecting the maintenance of body glucose homeostasis. This insulin-regulated protein transports glucose into tissues; thus, it is pivotal for efficient glucose uptake (Huang & Czech, 2007).

This study showed that RA did not affect the mRNA expression of FAS and GLUT-4; however, it decreased the aP2 and LPL transcript levels when used at the 100 μM dose. There was also a lowered RESTN mRNA expression after treatment with RA at a concentration of 10 μM (Fig. 4A). ST down-regulated the mRNA expression of FAS (decrease at a dose of 10 μM) and RESTN (decrease at doses of 10 μM and 100 μM) without affecting the aP2 and LPL gene expression. Whilst, ST at a concentration of 100 μM up-regulated GLUT-4 mRNA expression (Fig. 4B). STL significantly inhibited FAS, aP2, LPL and RESTN mRNA expression at all concentrations tested. Also, the mRNA expression of GLUT-4 was lowered at doses of 10 μM and 100 μM (Fig. 4C).

So far, only a limited number of *in vitro* studies have reported the effect of stevia-derived compounds on FAS, aP2, LPL and RESTN gene expression. FAS gene expression was down-regulated in 3T3-L1 adipocytes treated with ST at concentrations ranging from 50 μM to 200 μM (Park et al., 2022). Also, the RASE1 formulation containing RA and ST decreased FAS gene expression and other genes (aP2, LPL) involved in intracellular lipid metabolism. Moreover, RASE1 was found to inhibit GLUT-4 gene expression in the differentiating 3T3-L1 adipocytes (Khan et al., 2021). However, it should be noted that RASE1 is a mixture of green tea seed extract saponins and stevia extract compounds, and its activity is likely to be determined by other bioactive compounds combined with RA and ST in the formulation. In the research presented in this article, the inhibitory effect on GLUT-4 gene expression was evoked only by STL. In another study, treating 3T3-L1 cells with ST and STL led to up-regulation of GLUT-4 gene expression. These compounds at different doses enhanced GLUT-4 gene expression; namely, treatment with 100 μM ST and 1 μM STL caused the increased GLUT-4 transcript level in 3T3-L1 adipocytes (Bhasker et al., 2015). The same trend in stimulating GLUT-4 gene expression by supplementing with 100 μM ST is documented in the experiments presented in this article (Fig. 4B). Contrary, 3T3-L1 cell treatment with 1 μM STL did not affect GLUT-4 gene expression, whilst the STL at higher doses (10 μM and 100 μM) significantly decreased GLUT-4 transcript level (Fig. 4C). Due to the small number of studies, different experimental conditions and inconsistent data, further extensive investigations are needed to provide strong evidence of the inhibitory effects of SG on adipogenesis and lipogenesis.

Animal studies may provide valuable data to confirm the anti-adipogenic potential of SG in the management of obesity and obesity-

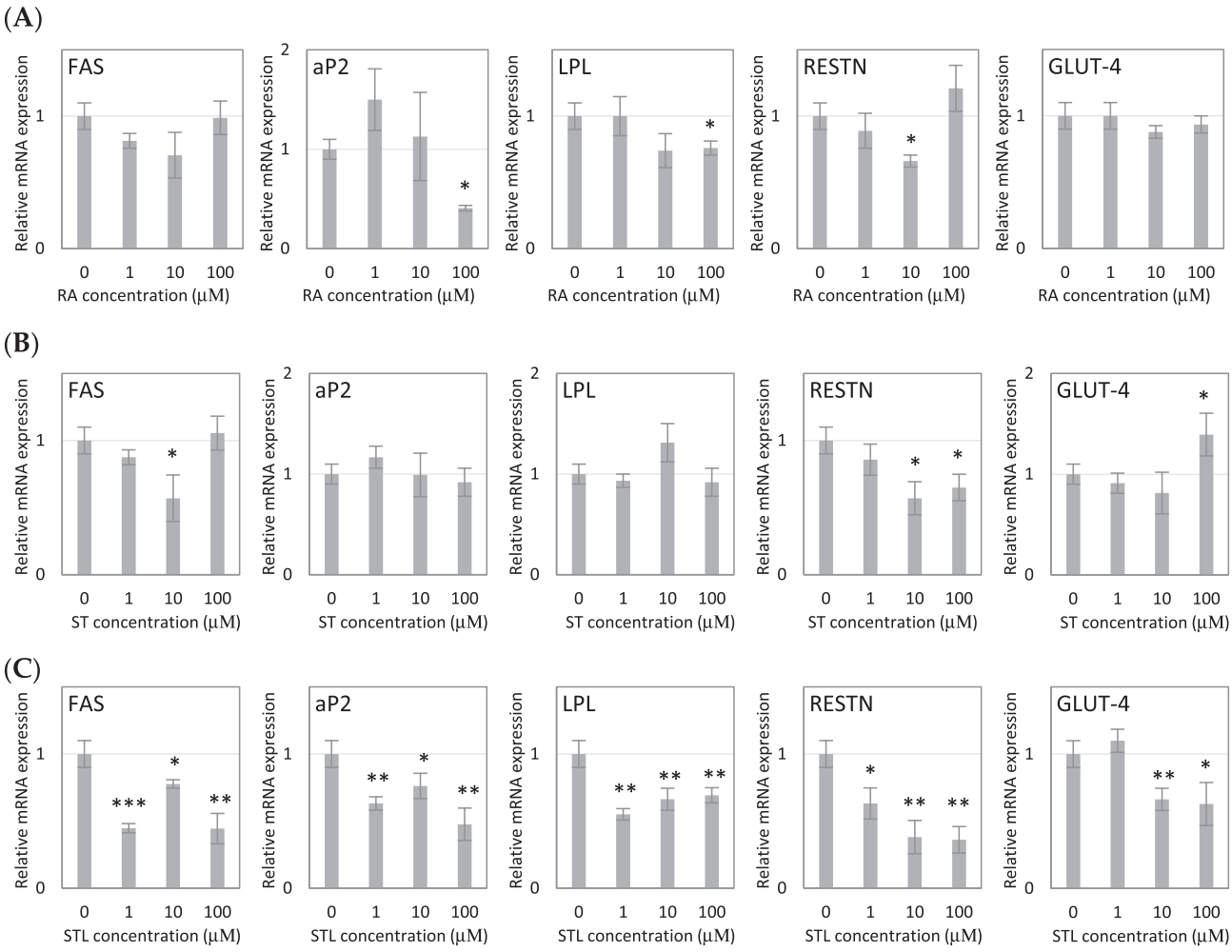

Fig. 4. Effect of RA (**A**), ST (**B**), and STL (**C**) on mRNA expression of genes involved in lipid metabolism (FAS, aP2 and LPL) and glucose metabolism (RESTN and GLUT-4) in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells stimulated by adipocyte differentiation medium without RA, ST, and STL treatment was the control (0). Data are shown as relative gene expression (fold of control). Significant differences at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ when compared with control (Tukey's post hoc test).

related disorders. The anti-adipogenic effects were documented in the epididymal adipose tissue of db/db mice following the oral ST administration. ST decreased epididymal adipocyte size but did not significantly reduce epididymal fat (Park et al., 2022). Animal experiments also indicated SG potential to improve the lipid profile and other health indices associated with excess body weight (Khan et al., 2021; Kurek et al., 2020; Ranjbar et al., 2020). Oral administration of SG combined with green tea saponins (RASE1 formulation) reduced mouse body and body fat pad weight and blood cholesterol, TG, glucose and insulin levels. RASE1 suppressed adipogenic and lipid metabolism gene expression in mouse adipose and liver tissues (Khan et al., 2021). The bioactivity of stevia derivatives is probably related to their inhibitory effects on the expression of adipogenic signaling molecules and genes involved in lipid metabolism.

3.4. The effect of steviol and steviol glycosides on leptin and adiponectin expression in 3T3-L1 adipocytes

This study evaluated the effect of stevia derivatives on the expression of leptin and adiponectin - cytokines involved in the metabolism of lipids and glucose secreted by adipose tissue. Leptin plays a pivotal role in stimulating food-seeking and determines various eating behaviours. Elevated blood leptin levels correlate with obesity and insulin resistance (Zhang & Chua, 2017). Mechanisms underlying the function of adiponectin are not fully understood, but it is known that it has pleiotropic properties. Adiponectin exhibits anti-inflammatory and anti-fibrotic properties, but its most important physiological role is to regulate insulin sensitivity. Decreased levels of adiponectin are commonly observed in diabetes and obesity (Fang & Judd, 2018).

The experiment showed that treatment with RA at a concentration of 100 μM reduced LEP mRNA expression and leptin protein secretion. A similar inhibitory effect of RA on adiponectin expression was documented at molecular and cellular levels (Fig. 5A). In contrast, ST did not affect LEP and ADIPOQ mRNA and protein expression (Fig. 5B). The effects of STL on adipokine expression were most pronounced. 3T3-L1 cell treatment with 10 μM and 100 μM of STL decreased mRNA LEP and ADIPOQ expression and lowered leptin and adiponectin protein secretion (Fig. 5C).

The results of studies investigating the effect of STL and SG on the expression of genes responsible for adipokine secretion and their actual concentrations are inconclusive. Studies in 3T3-L1 cell cultures and animal models showed that treatment with ST might increase adiponectin levels (Holvoet et al., 2015; Jayakumar, 2018; Khan et al., 2021). On the other hand, some reports indicate that ST does not significantly affect adiponectin and leptin concentrations in murine plasma (Kim et al., 2017). Although recent studies displayed that ST significantly decreased LEP mRNA gene expression in 3T3-L1 adipocytes (Park et al., 2022). Also, ST combined with RA and green tea saponins downregulated LEP gene expression in 3T3-L1 adipocytes and lowered leptin levels in serum and adipose tissues in an animal model (Khan et al.,

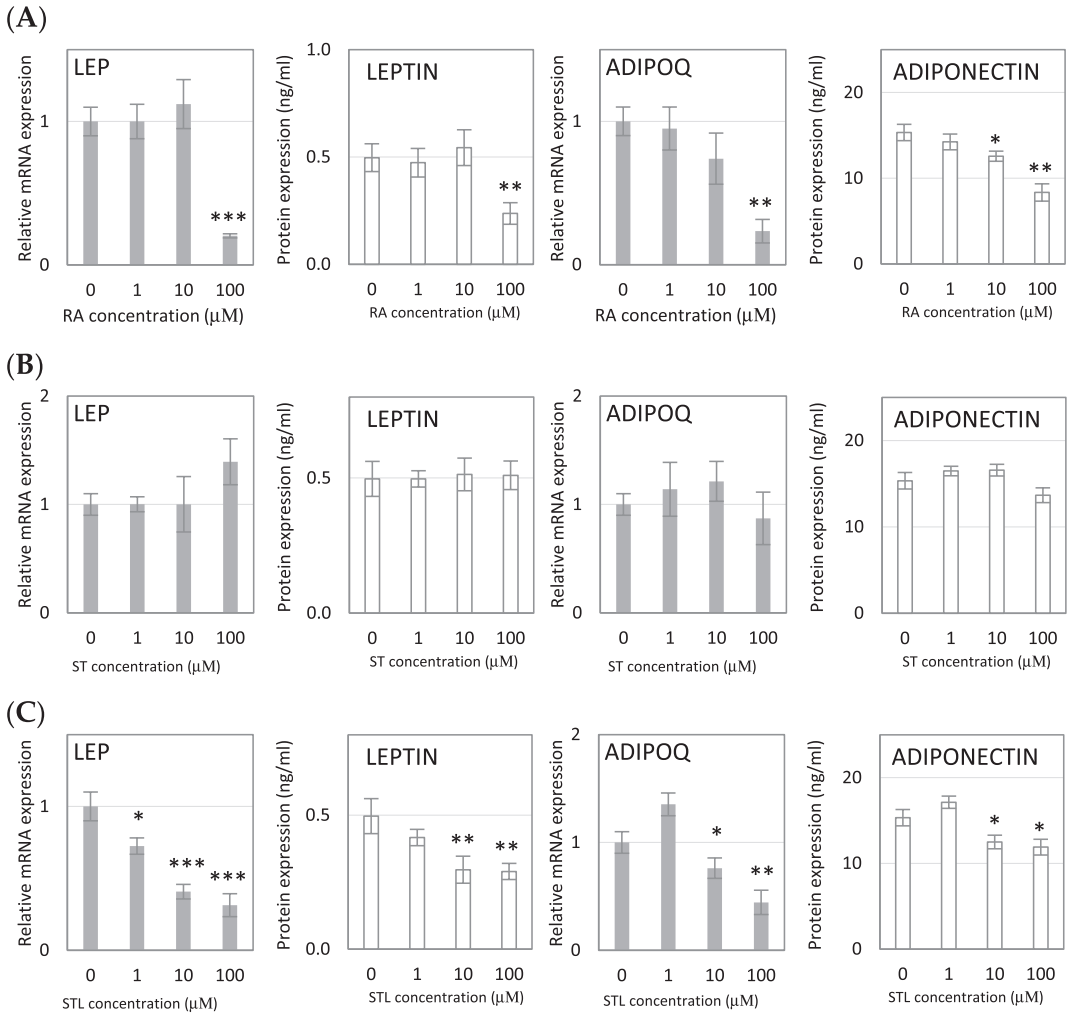

Fig. 5. Effect of RA (**A**), ST (**B**), and STL (**C**) on leptin (LEP) and adiponectin (ADIPOQ) mRNA expression and adipokine secretion in 3T3-L1 adipocytes. Differentiated 3T3-L1 cells stimulated by adipocyte differentiation medium without RA, ST, and STL treatment was the control (0). mRNA expression data are shown as relative gene expression (fold of control). Significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control (Tukey's post hoc test).

2021).

The inhibitory effect of STL on the expression of adipokines was noted in the present *in vitro* study and mice experiments conducted by Holvoet et al. (2015) (Holvoet et al., 2015). Scientists found that 12 weeks of STL administration (4 mg/kg body weight/day) caused a decrease in adiponectin blood level (1.8 ± 0.7 pg/mL vs 3.0 ± 2.1 pg/mL in the control group). Although a reduction in leptin expression may positively affect excessive adipogenesis, lowering the adiponectin level is not indicative of favourable health outcomes and may be related to, among others, increasing problems with insulin resistance.

3.5. The effect of steviol and steviol glycosides on glucose uptake, mRNA expression of genes involved in glucose metabolism and adipokine expression in insulin-resistant 3T3-L1 adipocytes

Recent findings indicate that compounds isolated from stevia may significantly affect carbohydrate-lipid metabolism, especially under hyperglycaemia conditions (Abudula et al., 2008; Naghiaee et al., 2020). Therefore, the impact of STL and SG on glucose uptake and glucose metabolism-related gene expression was examined in hypertrophic insulin-resistant adipocytes. The effect of the tested compounds on GLUT-4, RESTN, LEP, ADIPOQ mRNA expression and secretion of adipokines (leptin and adiponectin) was investigated in an insulin-resistant 3T3-L1 adipocyte model. RGZ (10 μM), a common drug applied in type 2 diabetes, was used as a positive control.

In the first step, cytotoxicity tests were performed, which revealed that none of the stevia compounds demonstrated cytotoxicity at concentrations ranging from 1 μM to 1000 μM, except STL, that reduced cell viability by approximately 50% at a dose of 1000 μM (Fig. 6A, 6B, 6C).

RA across concentrations ranging from 1 to 1000 μM significantly down-regulated the RESTN mRNA expression. RA at all doses tested also effectively reduced LEP gene expression and leptin secretion. In addition, adiponectin expression was suppressed by RA, except for the lowest concentration (1 μM). It is noteworthy that the inhibitory effects of RA and RZG on the expression of genes and proteins analysed in 3T3-L1 adipocytes were comparable. In contrast to RZS, RA did not effectively stimulate glucose uptake in insulin-resistant adipocytes. Moreover, after RA treatment, a down-regulating effect on GLUT-4 mRNA expression was observed (Fig. 6A).

In the insulin-resistant adipocyte model, ST showed a biological activity different from RA. Application of ST at doses of 1 μM and 10 μM resulted in increased GLUT-4 mRNA expression and improved glucose uptake. On the other hand, ST at the highest concentration (1000 μM) significantly down-regulated RESTN, LEP and ADIPOQ gene expression and decreased leptin and adiponectin secretion (Fig. 6B).

STL at a dose of 1000 μM induced cytotoxic effects in 3T3-L1 adipocytes; therefore, its beneficial health potential could not be considered at this concentration (Fig. 6C). STL at the lowest dose of 1 μM was effective in improving glucose uptake, up-regulating GLUT-4 mRNA expression and down-regulating RESTN mRNA expression, but without significantly affecting leptin and adiponectin secretion. The inhibitory effects on LEP and ADIPOQ mRNA expression and protein product secretion were more pronounced at higher doses of STL. The inhibitory effect of RZG on the adiponectin and leptin gene and protein expression is also worth noting. The lowering potential of the antidiabetic drug and STL and its glycosides were similar in potency (Fig. 6A, 6B and 6C). Equal efficiency in improving glucose uptake was observed after treating insulin-resistant 3T3-L1 adipocytes with ST (1 μM and 10 μM), STL (1 μM) and RZS (10 μM) (Fig. 6B and 6C).

Despite limited studies investigating the effect of STL and SG on insulin-resistant adipocytes, the results obtained show that ST can significantly improve glucose uptake regardless of insulin resistance (Mohd-Radzman et al., 2013). This effect has also been confirmed under treatment with STL but has not been investigated under insulin resistance conditions (Bhasker et al., 2015; Mohd-Radzman, 2013). Reports on the influence of these compounds on glucose uptake in studies using other experimental *in vitro* and *in vivo* models also show a significant effect of stevia-derived compounds, especially ST, on the improvement of glucose uptake (Bayat et al., 2020; Chen et al., 2007; Dandin et al., 2022; Deenadayalan et al., 2021; Prata et al., 2017; Rizzo et al., 2013).

Data from recent studies provide more and more evidence of the hypoglycaemic effects of ST, which is probably the most potent SG. Oral administration of diabetic Wistar rats with ST (20 mg/kg/day for 45 days) improved glucose and insulin tolerances. The treatment restored their elevated levels of fasting blood glucose, serum insulin and lipid profile to normalcy. Moreover, ST increased glucose uptake in diabetic gastrocnemius muscles by enhancing GLUT-4 synthesis, elevating binding affinity with IRS-1 and GLUT-4, and activating IR/IRS-1/Akt/GLUT-4 pathway (Deenadayalan et al., 2021). Beneficial effects of ST were also observed in a diet-induced obese zebrafish model, in which ST alleviated the hyperglycaemia and glucose intolerance, oxidative stress and insulin resistance associated with obesity (Dandin et al., 2022). SG hypoglycaemic potential was also documented for SG-riched formulations, such as commercial GlucoMedix® extract from *Stevia rebaudiana* and *Uncaria Tomentosa*. The GlucoMedix® exerted an anti-hyperglycemic effects in the alloxan-induced rats when it was administrated orally at doses of 250, 500, and 1000 mg/kg of body weight. In addition, GlucoMedix® reduced hyperlipidemia and hypertension in animal models (Villegas Vílchez et al., 2022).

Clinical trials have shown that ingested SG, ST and RA, are metabolized by the colon microbiota to the free STL compound (Mathur et al., 2017), assuming that ST and RA are converted to the steviol equivalents: STL-1.00; ST-0.395; RA-0.329. (Kubica et al., 2015). Intestinal STL absorption of up to 90% has been found after oral administration of SG (Geuns et al., 2007; Mathur et al., 2017; Wingard et al., 1980). Efficient intestinal absorption and high bioavailability of STL are necessary to induce health-promoting systemic effects.

The number of clinical trials investigating the potential of SG to improve the health of people struggling with lipid or carbohydrate problems published to date seems to be insufficient, as their results are often inconclusive. Some reports show that RA and ST exert no effects on lipid profile, glucose homeostasis or bodyweight (Barriocanal et al., 2008; Higgins & Mattes, 2019; Maki et al., 2008; Shin et al., 2016). However, Gregersen et al. (2004) found that an acute administration of 1,000 mg of ST significantly lowers the postprandial increase in glucose level in type 2 diabetic subjects (Gregersen et al., 2004). A study conducted by Ferri et al. (2006) showed that ST taken for 24 weeks at a dose of 15.0 mg/kg per day could improve the lipid profile, namely total cholesterol and low-density lipoprotein cholesterol in patients with hypertension (Ferri et al., 2006). Thus, the current state of knowledge indicates that SG might have significant potential to alleviate abnormalities in metabolic disorders.

STL-based treatment led to the most pronounced effect, suggesting that this compound may be involved in the health benefits evoked by ST and RA. Also, ST shows a more pronounced impact than RA, as it contains more STL per weight (Prakash et al., 2014). Moreover, STL can influence the expression of all signaling molecules and mediators analysed, which could be affected by RA and ST individually. On the other hand, there are no known genes whose expression is influenced by SG and not by STL (Holvoet et al., 2015). In the present study, the potential of ST and STL to improve glucose uptake and up-regulate GLUT-4 mRNA expression was shown in insulin-resistant 3T3-L1 adipocytes; however, the effect was closely dependent on the concentrations used. It is worth mentioning that the ambiguous and not strictly dose-dependent effects of using STL and SG have already been observed. Many studies have shown that lower doses of these compounds may bring more desirable results than higher doses. However, STL and SG did not become toxic as their concentrations increased, only their benefits diminished. This phenomenon shows that the mechanisms of action of stevia-derived compounds are still unexplored and require more extensive research to explain them in detail (Bhasker et al., 2015; Jeppesen et al., 2000; Mohd-Radzman, 2013; Mohd-Radzman et al., 2013).

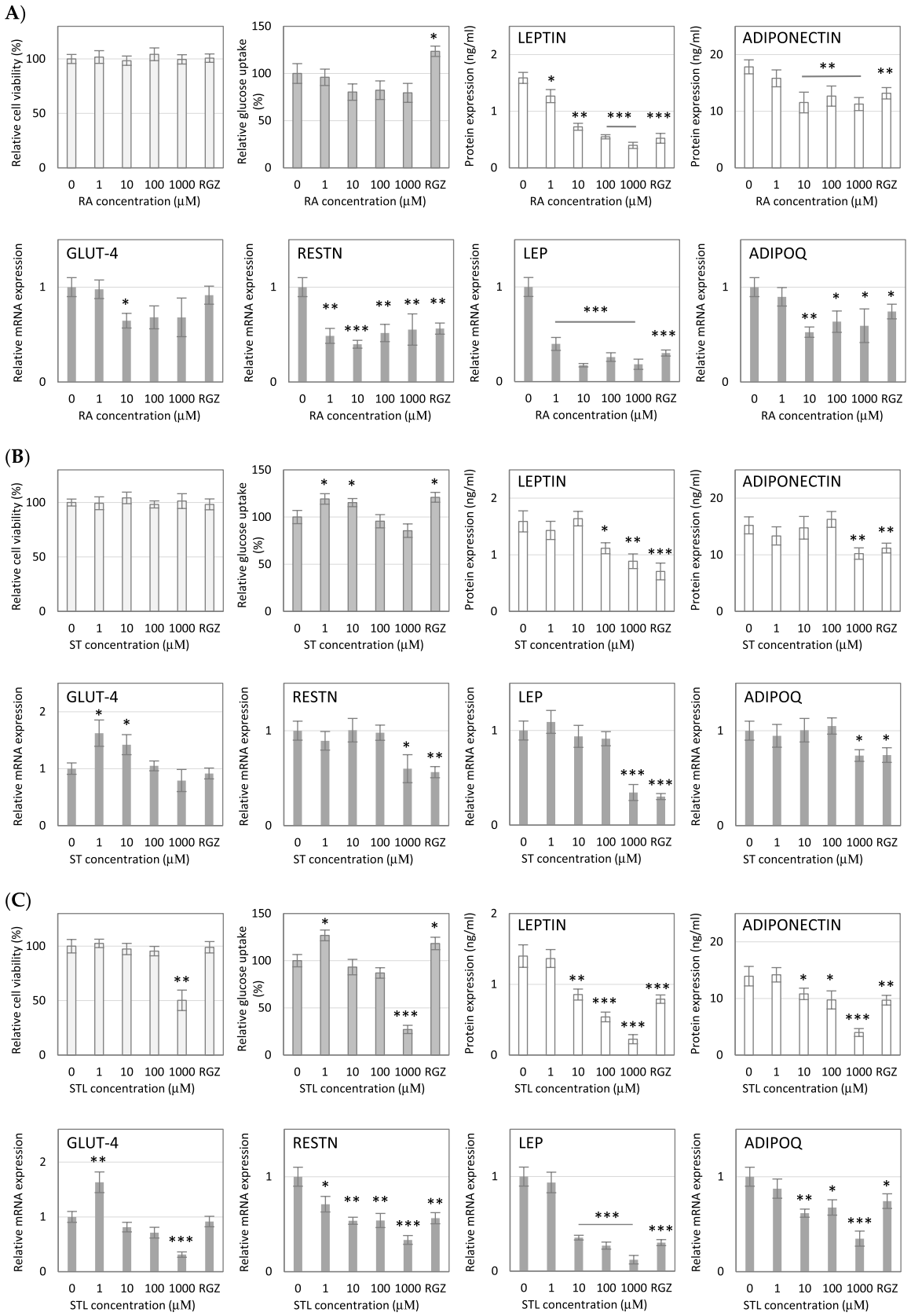

Fig. 6. Effect of RA (**A**), ST (**B**), and STL (**C**) on glucose uptake and mRNA expression of genes involved in glucose metabolism (RESTN and GLUT-4), and adipokine expression (LEP and ADIPOQ) in mature insulin-resistant 3T3-L1 adipocytes. As a positive control, RZG at a concentration of 10 μM was used in the experiment. mRNA expression data are shown as relative gene expression (fold of control). Significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control (Tukey's post hoc test).

4. Conclusions

This study showed that STL, a product of the metabolism of SG in the colon, is the most active tested compound capable of adipogenesis, lipogenesis and insulin resistance modulation. STL suppressed adipogenesis through down-regulation of PPARγ and C/EBPα transcription factors that activate the expression of aP2, LPL, fatty acid transporter and other genes significant for adipocyte phenotype creation. Lowered PPARγ and C/EBPα expression affected down-regulation of target LPL and aP2 gene expression. STL also inhibited the expression of the SREBP1 transcription factor, which stimulates the expression of many lipogenic genes, including FAS, leading to lipogenesis suppression. Moreover, STL displayed the potential to improve glucose uptake and alleviate insulin resistance probably by modulation of GLUT-4 and RSTN gene expression in insulin-resistant adipocytes. The promising data obtained make it possible to design further experiments to elucidate the mechanisms of action of steviol and its derivatives on various signaling pathways involved in adipogenesis, lipogenesis, and insulin resistance.

Given the bioavailability of STL, its anti-adipogenic potential and its ability to alleviate insulin resistance and regulate glucose uptake, SG as the metabolic sources of STL, may be proposed as natural free-calories sweeteners and functional food ingredients that can mitigate adverse effects of obesity and obesity-related disorders. However, further extensive *in vitro* and *in vivo* studies should be conducted to precisely describe the molecular mechanisms, cellular effects and physiological efficacy of SG administration. Also, strong evidence from clinical trials is needed to support the hypothesis about the usefulness of SG and STL for alleviating metabolic dysfunctions.

Ethical statement

In work, no animal and human experiments were performed. All research was conducted in accordance with abiding ethics regulations and guidance.

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CRediT authorship contribution statement

Jakub Michał Kurek: Conceptualization, Formal analysis, Investigation, Writing – original draft. **Joanna Zielińska-Wasielica:** Investigation. **Katarzyna Kowalska:** Methodology, Validation, Resources. **Zbigniew Krejpcio:** Conceptualization, Supervision, Project administration, Funding acquisition. **Anna Olejnik:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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