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Palmitate-increased TGF-β1 Gene Expression and p-Smad2/3 Protein Levels Attenuated by Chicoric Acid in Patients with Type 2 Diabetes Mellitus

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Abstract

Background: The transforming growth factor beta1 (TGF-β1) signaling pathway plays a critical role in the pathogenesis of Type 2 diabetes mellitus (T2DM). Modulating this pathway may offer therapeutic benefits for managing T2DM. Chicoric acid (CA), a polyphenolic compound with reported anti-diabetic properties, has shown potential in metabolic regulation; however, its precise molecular mechanisms remain unclear. This study aimed to investigate the effects of palmitate and CA on the TGF-β1 signaling pathway in peripheral blood mononuclear cells (PBMCs) from newly diagnosed T2DM patients and healthy controls.

Methods: A total of 40 participants, including 20 newly diagnosed T2DM patients and 20 age-matched healthy individuals (40–60 years), were enrolled in this study. Peripheral blood mononuclear cells (PBMCs) were isolated and treated with palmitate and CA. The expression of TGF-β1 mRNA was analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Protein levels of Smad2/3 and phosphorylated Smad2/3 (p-Smad2/3) were assessed via western blot analysis.

Results: Palmitate stimulation significantly upregulated TGF-β1 gene expression and increased p-Smad2/3 protein levels in PBMCs. However, CA treatment effectively attenuated these palmitate-induced elevations in TGF-β1 expression and p-Smad2/3 protein levels. Additionally, a positive correlation was observed between TGF-β1 expression and p-Smad2/3 protein levels.

***Conclusion*s:** These findings suggest that CA may act as an inhibitor of the TGF-β1 signaling pathway, potentially contributing to T2DM management by downregulating TGF-β1/Smad signaling. Further studies are warranted to explore its therapeutic potential in diabetes treatment.

Keywords: Chicoric acid, Palmitate, Smad signaling, Transforming growth factor-beta 1, Type 2 diabetes mellitus.

Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disease that has become a significant global public health concern. Its prevalence is steadily increasing, with projections indicating that by 2030, approximately 7,079 individuals per 100,000 will be affected worldwide (1). A key characteristic of T2DM is insulin resistance (IR), which is influenced by various factors, including chronic inflammation (2, 3). Peripheral blood mononuclear cells (PBMCs), composed mainly of lymphocytes and monocytes, serve as an effective model for

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evaluating inflammatory responses in T2DM patients (4, 5).

The transforming growth factor-beta (TGF-β) superfamily consists of multiple proteins involved in essential cellular processes such as proliferation, differentiation, and apoptosis. More than 30 members of this superfamily have been identified in humans, including TGF-βs, activins, inhibins, growth differentiation factors, and bone morphogenetic proteins (BMPs). Among them, TGF-β1 plays a pivotal role in diabetes pathophysiology (6, 7). As a pro-inflammatory cytokine, TGF-β1 contributes to IR by binding to its receptors (TβRI and TβRII), initiating a phosphorylation cascade that activates Smad signaling. The phosphorylated Smad2/3 (p-Smad2/3) complex subsequently translocate to the nucleus, where it regulates gene expression (6, 8-10). Studies suggest that the TGF-β1/Smad pathway is deeply involved in metabolic disorders such as obesity, IR, and T2DM (11-13). This pathway influences glucose and lipid metabolism, insulin secretion in pancreatic β-cells, adipocyte differentiation, white adipose tissue (WAT) browning, and the regulation of oxidative stress and inflammation. Numerous studies have highlighted the pro-inflammatory role of TGF-β1 (14), while Smad2/3 deficiency has been associated with reduced inflammation and improved IR in adipose tissue. Thus, targeting the TGF-β1/Smad pathway presents a promising therapeutic strategy for T2DM management (15).

Elevated circulating levels of free fatty acids (FFAs), particularly palmitate, are often observed in individuals with IR and T2DM. Chronic exposure to palmitate has been shown to exacerbate oxidative stress, promote inflammation, and increase p-Smad2/3 protein levels, suggesting that it may contribute to IR via activation of the TGF-β1/Smad signaling pathway (16). Chicory (Cichorium intybus L.) is widely recognized for its medicinal properties and has traditionally been used in the treatment of various diseases, including T2DM (17, 18). Chicoric acid (CA), a polyphenolic compound extracted from Chicory, exhibits anti-inflammatory, antioxidant, and anti-hyperglycemic properties. However, the precise molecular mechanisms underlying these effects remain incompletely understood (19). Given the potential role of TGF-β1 in IR and inflammation, this study aims to investigate the effects of palmitate and CA on TGF-β1 gene expression and Smad2/3 signaling in PBMCs from newly diagnosed T2DM patients and healthy individuals.

Materials and Methods

Study design and biochemical analysis

This study included 20 newly diagnosed patients with type 2 diabetes mellitus (T2DM) and 20 healthy individuals, aged 40 to 60 years (mean age: 47.23 ± 7.41 years). Participants were recruited from individuals attending Hamadan Health Center. The diagnosis of T2DM was established based on the latest criteria set by the American Diabetes Association (ADA) (20).

Participants aged between 40 and 60 years, newly diagnosed T2DM patients (without prior use of anti-diabetic medication), and healthy controls with no history of diabetes were included in the study. Individuals with acute or chronic inflammatory diseases, cardiovascular disease, uncontrolled hypertension, type 1 diabetes mellitus (T1DM), gestational diabetes, smoking habits, or alcohol consumption were excluded.

The study protocol received ethical approval from the Ethics Committee of Hamadan University of Medical Sciences (approval code: IR.UMSHA.REC.1397.501). All participants provided written informed consent prior to the study.

Following a 12-hour fasting period, anthropometric measurements, including height, weight, body mass index (BMI: weight/height^2), systolic blood pressure (SBP), and diastolic blood pressure (DBP), were recorded. Subsequently, 20 mL venous blood samples were collected for biochemical analysis and PBMC isolation.

Biochemical parameters, including fasting blood sugar (FBS), triglycerides (TG), total

cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, and creatinine, were assessed using colorimetric methods with commercial kits (Pars Azmoon, Tehran, Iran) on a BIOLIS24i Premium autoanalyzer (Tokyo Boeki Machinery Ltd., Japan). Serum insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Monobind Inc., CA, USA). Insulin resistance was evaluated using the homeostasis model assessment of insulin resistance (HOMA-IR) formula: fasting insulin (µIU/ml) × FBS (mg/dl) / 405. Hemoglobin A1C (HbA1C) was analyzed using high-performance liquid chromatography (HPLC) with a Tosoh G8 instrument (South San Francisco, CA, USA).

Lymphocytes isolation and treatment

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from heparinized whole blood samples immediately using Ficoll-Hypaque density-gradient centrifugation under sterile conditions (Lympholyte-H; Cedarlane Laboratories, Ontario, Canada) as previously described (21). After washing the cells twice with phosphate-buffered saline (PBS), isolated PBMCs suspended in RPMI 1640 medium (GIBCO; Invitrogen Laboratories, UK). Then PBMCs were counted, and viability of cells was evaluated by trypan blue staining. 2×10^6 cells/well and 5×10^6 cells/well plated in 12-well plate and 6-well plate containing RPMI 1640 supplemented with 10% heat inactivated FBS (fetal bovine serum) (GIBCO; Invitrogen Laboratories, UK) and 1% penicillin-streptomycin (GIBCO; Invitrogen Laboratories, UK) for gene expression and western blotting analysis, respectively.

Sodium palmitate (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 50% (v/v) ethanol by heating at 55 °C and then diluted 1:100 in RPMI 1640 medium containing 1% (w/v) fatty acid free-bovine serum albumin (BSA). BSA-conjugated palmitate was prepared after two h shaking in incubator under 37 °C. CA (Sigma Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). For sterilization, solutions were passed through 0.2 filters.

After two h incubation under 37 °C with 5% CO2, PBMCs were treated as follows: Control groups (untreated, treated with BSA 1% for 12 h), CA groups (treated with 50 μM CA for 6 h), Palmitate groups (treated with 500 μM palmitate for 12 h), Palmitate + CA groups (treated with 500 μM palmitate for 12 h and then treated with 50 μM CA for 6 h). Finally, the cells were harvested and stored at −80 °C for evaluation of TGF-β1 gene expression and measuring Smad2/3 and p-Smad2/3 protein levels. It should be noted that optimum concentration and exposure time for CA and palmitate treatment were used according to the results of previous studies (5, 21).

Quantitative-real time PCR (qRT-PCR)

Total RNA was extracted from the treated PBMCs with a Hybrid-R RNA purification kit (Gene All Biotechnology, Seoul, Korea) and reverse transcribed using Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Thermo Scientific, Fermentas, USA). Quantity and integrity of extracted RNA was evaluated by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and 1% agarose gel electrophoresis, respectively. Real-time PCR was performed in a light cycler 96 (Roche Diagnostics, Germany) using SYBR Green detection kit (Ampliqon, Denmark) and gene-specific primers. Primer sequences were as follows: β-actin (NM_001101.4): 5′-ACAGAGCCTCGCCTTTGC-3′ (forward) and 5′-ATCACGCCCTGGTGCCT-3′ (reverse); TGF-β1 (NM_000660.7): 5′-ATTCCTGGCGATACCTCAGCA-3′ (forward), 5′-GTAGTGAACCCGTTGATGTCC-3′ (reverse). β-actin was used as the reference gene. Fold change in TGF-β1 gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

Isolated PBMCs were homogenized in RIPA buffer (Thermo Scientific, Waltham, MA,

USA) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Protein concentrations were determined using the bicinchoninic acid (BCA) method (BCA Protein Assay Kit, Thermo Fisher Scientific Inc., USA). Equal amounts of each sample (70 µgr total proteins) were run on a 10% SDS-polyacrylamide gels. After separation, protein samples were transferred to 0.22 µm pore nitrocellulose membrane. The membranes were blocked with Tris-buffered saline, 0.1% Tween 20 (TBST) containing 5% non-fat milk for two h at room temperature and then incubated with primary antibodies against β-actin, Smad2/3 and p-Smad2/3 (Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing three times with TBST, membranes incubated for one hour at room temperature with goat anti-rabbit IgG HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA). Finally, immunoreactivity was visualized with Enhanced chemiluminescence (ECL) emitting kit (Bio-Rad, Germany). Protein bands intensity were quantified using Image J software (NIH, Bethesda, USA).

Statistical analysis

All data were expressed as mean ± standard deviations (SD). Statistical analysis was performed using Two-way analysis of variance (Two-way ANOVA) using SPSS software (version 16.0, Chicago, IL, USA) and GraphPad Prism software (version 7, San Diego, CA, USA). Pearson's correlation test was used to evaluate correlation between variables. The p value <0.05 was regarded as statistically significant.

Results

Effects of palmitate and CA on TGF-β1 gene expression

The TGF-β1 gene expression was significantly increased in PBMCs treated with palmitate compared with untreated cells by 2.21 and 2.66-fold in healthy subjects and patients with T2DM, respectively ($p< 0.001$) (Fig. 1).

However, TGF-β1 gene expression significantly down regulated by CA in PBMCs of healthy subjects (0.61-fold) and patients with T2DM (0.68-fold) ($p< 0.05$). In addition, palmitate-induced TGF-β1 expression was reduced by CA to 1.34-fold and 1.78-fold in healthy volunteers and patients with T2DM, respectively ($p< 0.001$). The assessment of TGF-β1 gene expression in untreated cells showed that there was no significant difference between healthy and diabetic groups.

Effects of palmitate and CA on Smad2/3 and p-Smad2/3 protein levels

The results failed to indicate significant difference in Smad2/3 protein levels between groups (Fig. 2). However, p-Smad2/3 protein level was significantly increased in response to palmitate in PBMCs of healthy control group (1.67-fold) and diabetic group (1.65-fold) compared to untreated cells ($p< 0.001$). Unlike palmitate, incubating the PBMCs with CA decreased p-Smad2/3 protein level with respect to the untreated PBMCs in healthy subjects and patients with T2DM approximately 0.54 and 0.49-fold, respectively ($p< 0.01$). Also, we found that CA was able to restore palmitate-increased p-Smad2/3 protein level to near control in healthy subjects (from 1.67-fold to 1.28, $p = 0.019$). Furthermore, the between group analysis revealed that p-Smad2/3 protein levels in untreated cells was lower in healthy control compared to the patients with T2DM however it was not significant (Fig. 3).

Association between TGF-β1 gene expression with Smad2/3 and p-Smad2/3 protein levels

No association was observed between TGF-β1 gene expression and Smad2/3 protein levels. However, analyzes of correlation between TGF-β1 gene expression and p-Smad2/3 protein levels revealed a strong positive association between these variables in untreated cells ($r = 0.832$, $p = 0.003$) and CA-induced cells ($r = 0.420$, $p = 0.029$) from patients with T2DM (Table 1).

Table 1. Correlation coefficients of TGF-β1 gene expression with Smad2/3 and p-Smad2/3 protein levels in each group.

		TGF-β1 gene expression (r)			
		Untreated PBMCs	**Chicoric acid treated PBMCs**	**Palmitate treated PBMCs**	**Palmitate + Chicoric acid treated PBMCs**
Smad2/3 protein levels	Healthy subjects	0.538	0.421	0.223	0.562
	Patients with T2DM	0.064	0.810	0.502	0.355
p-Smad2/3 protein levels	Healthy subjects	0.637	0.030	-0.170	-0.947
	Patients with T2DM	**0.832**	**0.420**	0.042	0.495

Bold texts are statistically significant. *p< 0.05.

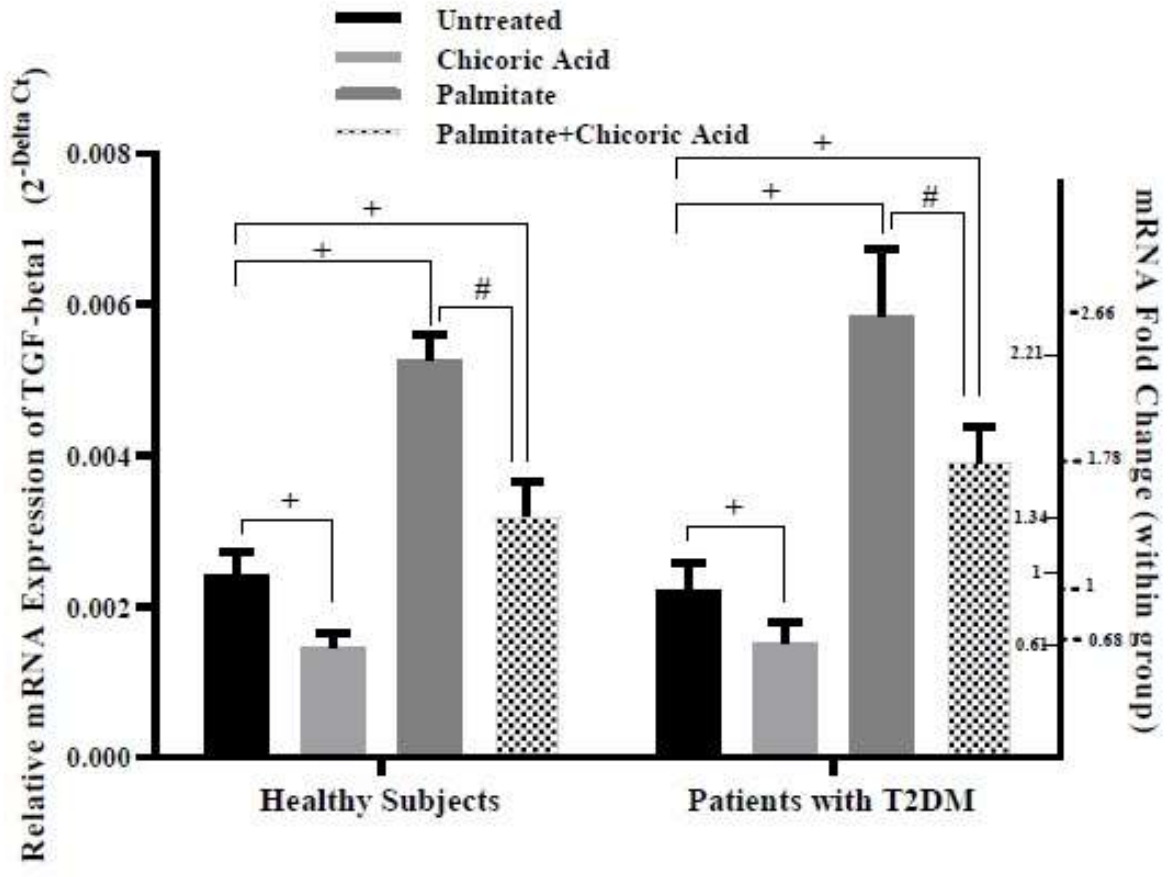

Fig. 1. Effects of palmitate and CA on TGF-β1 gene expression in PBMCs of healthy subjects and patients with T2DM. Left side of the graph shows mRNA expression ($2^{-\Delta Ct}$) and right side of the graph shows mRNA-fold change ($2^{-\Delta\Delta Ct}$). The non-dotted and dotted lines show-fold change for healthy subjects and patients with T2DM, respectively. Data are shown as mean ± SD. ^{+}p< 0.05 compared to untreated cells, $^{\#}p$< 0.05 compare to palmitate treated cells.

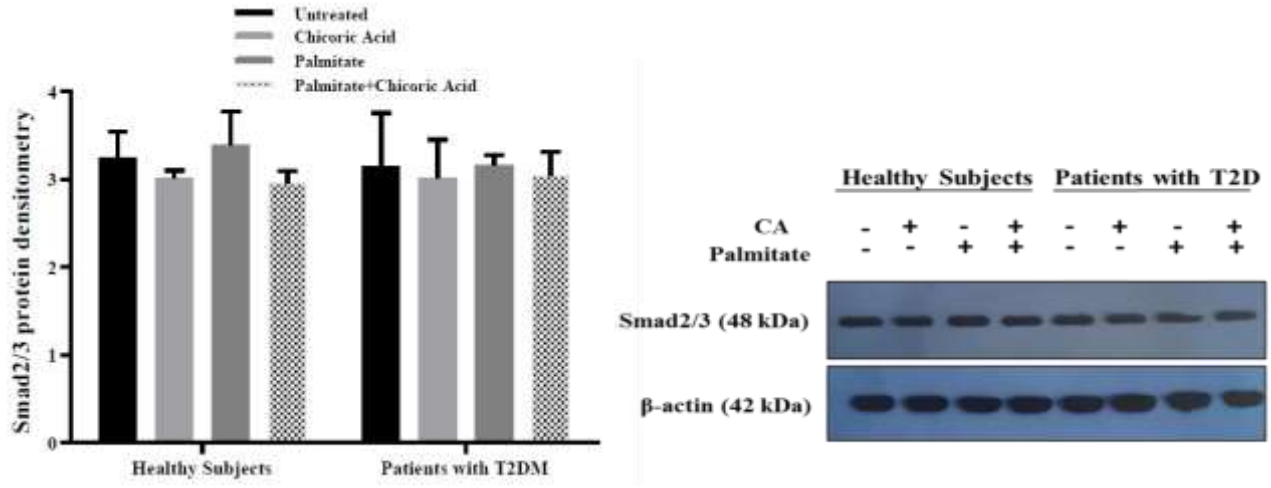

Fig. 2. Effects of palmitate and CA on Smad2/3 protein densitometry in PBMCs of healthy subjects and patients with T2DM. Western blotting bands are representative of one sample per group for each group and graphs are representative of twenty samples per group for each group. The band intensity was quantified densitometrically using the ImageJ software (National Institutes of Health, Bethesda, MD). Western blotting results were normalized to β-actin and are displayed as the mean ± SD.

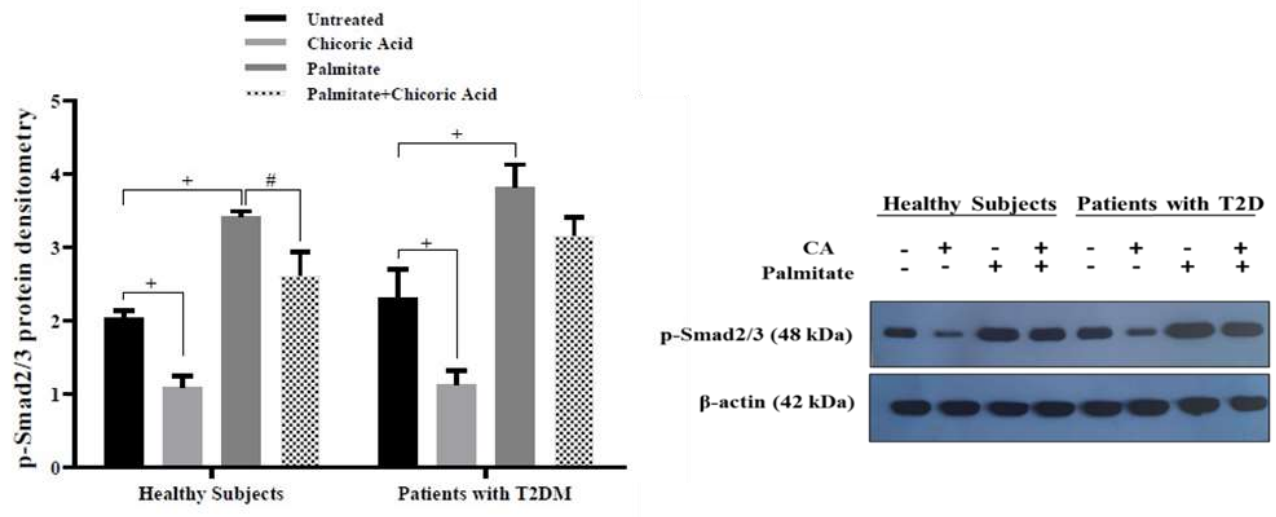

Fig. 3. Effects of palmitate and CA on p-Smad2/3 protein densitometry in PBMCs of healthy. subjects and patients with T2DM. Western blotting bands are representative of one sample per group for each group and graphs are representative of twenty samples per group for each group. The band intensity was quantified densitometrically using the ImageJ software (National Institutes of Health, Bethesda, MD). Western blotting results were normalized to β-actin and are displayed as the mean ± SD. $^{+}p< 0.001$ compared to untreated cells, $^{\#}p< 0.001$ compared to palmitate treated cells.

Discussion

We initially observed that the gene expression of TGF-β1 and the protein levels of Smad2/3 and phosphorylated Smad2/3 (p-Smad2/3) in untreated PBMCs of newly diagnosed T2DM patients were not significantly different from those of healthy subjects. The TGF-β1 signaling pathway has been widely implicated in the pathogenesis of T2DM, with several studies reporting elevated levels of TGF-β1-related proteins in diabetic patients (22, 23). For instance, Thomsen et al. (2017) found increased p-Smad levels in kidney tissues of T2DM patients, suggesting that persistent activation of the TGF-β1 pathway contributes to diabetic nephropathy (24). The discrepancy in findings could be attributed to disease duration; while prior studies focused on individuals with longstanding diabetes, our study included newly diagnosed patients. Since TGF-β1 is closely linked to diabetes-

related complications such as nephropathy and retinopathy (24, 25), its impact may become more pronounced over time. Chronic exposure to elevated insulin levels in T2DM has been shown to upregulate TGF-β1 receptor expression, ultimately promoting tissue fibrosis (26).

Our findings further indicate that palmitate treatment leads to a significant upregulation of TGF-β1 gene expression and p-Smad2/3 protein levels. These results align with previous research demonstrating the stimulatory effects of free fatty acids (FFAs) on TGF-β1 signaling. Nath et al. (2015) reported that palmitate activates the TGF-β1 pathway in HepG2 cells, reinforcing its role in metabolic dysfunction (27). Similarly, Sun et al. (2015) showed that palmitate exposure triggers the Smad3 signaling cascade in podocytes (16). Additionally, fatty acid synthase (FASN), a key enzyme in lipid metabolism, has been identified as an upstream regulator of TGF-β1 signaling (28). Recent studies also highlight that palmitate exacerbates insulin resistance by promoting inflammation via MyD88-dependent pathways (29). In line with the results of this study, palmitate also activates the TGF-β1 signaling pathway, which leads to increased inflammation and consequently contributes to IR. (15). Therefore, it is likely that palmitate induces IR by increasing inflammation in a MyD88 and TGF-β1-dependent signaling pathway.

To further elucidate the protective role of chicoric acid (CA) in T2DM, we examined its influence on TGF-β1 signaling components. Our study provides novel evidence that CA reduces both TGF-β1 gene expression and p-Smad2/3 protein levels. These findings are consistent with prior studies demonstrating that certain polyphenolic compounds, such as rosmarinic acid, downregulate TGF-β1 and Smad phosphorylation (30). In another study, Yadav et al. (2011) showed that Smad3-deficient mice exhibited lower inflammatory cytokine levels, improved insulin sensitivity, and increased mitochondrial content in white adipose tissue, suggesting a beneficial metabolic effect (31). Furthermore, blocking TGF-β1 signaling with neutralizing antibodies was found to reduce Smad3 phosphorylation, enhancing glucose and insulin tolerance. Collectively, these findings support the hypothesis that inhibiting the TGF-β1/Smad pathway could serve as a promising strategy for improving insulin resistance and metabolic health in T2DM.

An interesting observation in our study was that total Smad2/3 protein levels remained unchanged across all groups. This suggests that the phosphorylation status of Smad2/3, rather than its absolute expression, plays a pivotal role in mediating TGF-β1 signaling. Indeed, p-Smad2/3 serves as the key effector of this pathway, translocating to the nucleus upon TGF-β1 receptor activation to regulate target gene transcription (8). Moreover, we found a strong positive correlation between TGF-β1 expression and p-Smad2/3 levels, further confirming the central role of Smad phosphorylation in TGF-β1-mediated effects (8, 9).

Our findings suggest that CA functions as a novel inhibitor of the TGF-β1/Smad signaling pathway, which may contribute to its beneficial effects in T2DM. Given the established role of this pathway in diabetes complications, targeting TGF-β1 signaling could potentially mitigate the progression of diabetic nephropathy and other associated disorders. In summary, CA presents a promising therapeutic candidate for T2DM, although further research is necessary to fully elucidate its molecular mechanisms and long-term efficacy.

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Ethics

The study protocol was approved by the Ethics Committee of Hamadan University of Medical Sciences (code: IR.UMSHA.REC.1397.501).

Conflicts of interest

The authors declare no conflict of interest.

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