Original Research

Cholinergic activation suppresses palmitate-induced macrophage activation and improves acylation stimulating protein resistance in co-cultured adipocytes

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Impact statement

1. Adipocyte–macrophage interaction in acylation-stimulating protein (ASP) resistance
2. Lipotoxicity induced inflammatory response in ASP resistance
3. A vicious circle between lipotoxicity and inflammatory response in ASP resistance
4. Cholinergic modulation of inflammatory response in adipocyte and macrophage

Abstract

Acylation-stimulating protein (ASP), produced through activation of the alternative complement immune system, modulates lipid metabolism. Using a trans-well co-culture cell model, the mitigating role of α7-nicotinic acetylcholine receptor (α7nAChR)-mediated cholinergic pathway on ASP resistance was evaluated. ASP signaling in adipocytes via its receptor C5L2 and signaling intermediates Gαq, Gβ, phosphorylated protein kinase C-α, and protein kinase C-ζ were markedly suppressed in the presence of TNFα or medium from palmitate-treated RAW264.7 macrophages, indicating ASP resistance. There was no direct effect of α7nAChR activation in 3T3-L1 cell culture. However, α7nAChR activation almost completely reversed the ASP resistance in adipocytes co-cultured with palmitate-treated RAW264.7 macrophages. Further, α7nAChR activation could suppress the production of pro-inflammatory molecules TNFα and interleukin-6 produced from palmitate-treated co-cultured macrophages. These results suggest that macrophages play a significant role in the pathogenesis of ASP resistance and α7nAChR activation secondarily improves adipose ASP resistance through suppression of inflammation in macrophages.

Keywords: Acylation stimulating protein, α7-nicotinic acetylcholine receptor, obesity, adipokine, inflammation

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Introduction

Obesity continues to be a major public health problem. Numerous studies conducted thorough systematic reviews of obesity indicate that the number of obese individuals has never been larger worldwide than today.[1,2] Kelly *et al.* predict that overweight and obese populations will constitute a major proportion by 2030 when evaluated on a world scale.[3] Obesity is characterized by specific pathogenic entities, including excess adipose tissue, aberrant levels of circulating adipokines, imbalances in the adaptive immune system in adipose tissue, ectopic fat accumulation, and chronic systemic inflammation.[4]

Acylation-stimulating protein (ASP), as a by-product due to proximal activation of the alternative complement system, is an adipokine-stimulating lipid metabolism. In many cases, the effects of insulin (INS) and ASP are additive yet independent of each other.[5,6] ASP action is mediated through its receptor C5L2.[7,8] A dysfunction of ASP–C5L2 signaling involved in triglyceride synthesis (TGS) may contribute to induced INS resistance.[9] Dysfunction (or resistance) to both of these hormones can contribute to severe metabolic disturbances. On the other hand, in obese individuals, acute postoperative reduction in ASP levels is associated with early improvement of INS resistance after

biliopancreatic diversion with duodenal switch surgery.[10] This study suggested that restoration of ASP sensitivity precedes physiological restitution of INS sensitivity. Interestingly, both C3KO mice receiving chronic recombinant ASP and C5L2KO mice fed a high fat or diabetogenic diet, both examples of induced ASP resistance, are accompanied by onset of INS resistance and inflammation. Therefore, ASP–C5L2 pathway disruption may have deleterious metabolic effects which directly contribute to development of INS resistance and chronic inflammatory responses.[9,11,12]

Generally, increases in postprandial dietary fatty acids (FAs) result in a compensatory increase in ASP. However, these postprandial ASP increases are primarily detected locally in adipose tissue and not in the general circulation.[13] Based on cell studies, ASP generation is modulated by various inflammation-related factors and hormones, as well as drugs used in the treatment of metabolic disorders.[14–21] High levels of circulating ASP are a hallmark for abnormal lipid metabolism and dyslipidemia in obesity-associated comorbidities and have been shown to be associated with ASP resistance. At the level of adipose tissue, this could impede FA esterification and result in delayed triglyceride (TG) clearance. It is worthwhile to note that ASP also has immunoregulatory effects. *In vitro*, ASP stimulates chemokine secretion in adipocytes,[22] cytokine synthesis in human peripheral blood mononuclear cells[23] and tonsil-derived B cells,[24] and induces macrophage M1 phenotype polarization.[25] *In vivo*, ASP supplementation leads to inflammatory changes in tissue and plasma levels.[11] While reduced ASP function (ASP resistance) has been demonstrated for its lipid-modulating roles, the impact on its inflammation-related regulatory function has not been evaluated.

In adipose tissue from obese subjects, adipocyte dysfunction occurs and free fatty acids (FFAs), which flux from hypertrophied adipocytes, are capable of inducing INS resistance in liver, muscle, and adipose tissue,[26–28] while ASP resistance is induced mainly in adipose tissue.[11,18] Macrophages, among other cells, increasingly infiltrate into adipose tissue to scavenge adipocyte-derived lipids.[29] Paracrine cross talk between adipocytes and macrophages constitutes a vicious circle which continuously aggravates inflammatory change and metabolic disorder. Inflammation may be an important link between obesity and ASP resistance. Although the precise mechanism of ASP resistance is still not fully elucidated, the basic concept is analogous to INS resistance, which is defined by both functional resistance and molecular resistance. Studying potential anti-inflammatory mechanisms may provide novel therapeutic targets in the treatment of ASP resistance, dyslipidemia, and lipotoxicity.

The α7-nicotinic acetylcholine receptor (α7nAChR) pathway plays a key role in both central and peripheral mechanisms involved in eating behavior and energy homeostasis. A growing body of evidence has emerged showing that α7nAChR may be of pathophysiological relevance in obesity and associated complications.[30–34] Centrally, α7nAChR activation elicits appetite inhibition by affecting hypothalamic neuropeptides, multiple neurotransmitter systems, limbic and paralimbic systems in the brain. Peripherally, long-term vagal nerve stimulation or selective cholinergic agonists mimicking this effect reduce body weight and improve metabolic disorders by mediating anti-inflammatory effects.[35] Activation of α7nAChR pathways in adipocytes and macrophages mediates anti-inflammatory response through inhibition of inflammatory cytokine production.[32,33,36] Smokers or experimental animal models treated with nicotine have reduced risk of weight gain. Nicotine improved INS sensitivity in rodents but not in α7nAChR knockout animals.[32] The α7nAChR-mediated cholinergic anti-inflammatory pathway has beneficial effects on normalization of lipid and glucose metabolic parameters and improvement of INS sensitivity.[32] Nicotine supplementation in high-fat diet-induced obesity (DIO) mice results in increased serine phosphorylation of AKT in liver, skeletal muscle, and adipose tissue. AKT is one of the main intracellular response components in INS signaling. However, there is still a lack of direct evidence demonstrating a convergence of the cholinergic and INS pathways. The amelioration of the INS resistant state is usually accompanied by concomitant reduction in pro-inflammatory cytokines including TNFα, interleukin-6 (IL-6), interleukin-1β, and inducible nitric oxide synthase levels in α7nAChR-agonist-treated fatty rats[30] and db/db mice.[31,32] Both high levels of TNFα secretion from adipose tissue and serum IL-6 are associated with INS resistance.[37] Several lines of evidence indicate that TNFα can affect INS signal transduction through altering serine phosphorylation of AKT and PKC activation.[38–41] TNFα acts locally on adipose tissue in a paracrine or autocrine fashion. IL-6 has also been considered as a major circulating component in obesity-associated INS resistance. However, IL-6 effects on INS action appear to be controversial in liver, muscle, and adipose tissue. Nevertheless, it is reasonable to speculate that the cholinergic-mediated beneficial effects are mediated via inflammation pathways. Cholinergic activation could conceivably ameliorate INS resistance through altering profiles of pro-inflammatory cytokines in adipose tissue.

Normal expression levels and internalization of cell surface C5L2, balancing between recycling and degradation of cell surface and intracellular C5L2, are all necessary for ASP to activate a cellular response. In addition, G protein and protein kinase C (PKC) are crucial downstream mediators for ASP-induced TGS in adipocytes. Several other well-known signaling proteins in the ASP stimulation of TGS converge with the INS pathway including PDK, phosphoinositide 3-kinase, and AKT.[42,43] Kox *et al.*[44] demonstrated that GTS-21, an α7nAChR selective partial agonist is more effective than nicotine in modulating the immune response and may be more suitable for human *in vivo* trials to explore cholinergic function. In the present study, we ascertained the potential overlap of specific cytokines as possible mediators between the cholinergic pathway and ASP stimulation of TGS through treating a co-culture system with bovine serum albumin (BSA)-coupled long-chain saturated FA palmitate, mimicking an obese lipotoxicity–inflammation microenvironment.

Materials and methods

Regents

Murine 3T3-L1 preadipocytes and RAW264.7 macrophages were both purchased from American Type Culture Collection (Manassas, Virginia, USA). INS, isobutylmethylxanthine, dexamethasone (DEX), FFA-free BSA, palmitate, and GTS-21 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Antibodies used were as follows: Gα(q) rabbit monoclonal antibody (14373s, Cell Signaling, USA); Gβ rabbit polyclonal antibody (sc-378, Santa Cruz Biotechnology, California, USA); PKC alpha (phosphor-S657) rabbit monoclonal antibody (ab180848); PKC zeta (phosphor-T560) rabbit monoclonal antibody (ab59412); β-actin rabbit polyclonal antibody (ab8227) (Abcam, Shanghai Abcam Trading Company Ltd, Shanghai, China). Recombinant ASP was kindly purified by Marc Lapointe.[45]

Cell culture

3T3-L1 preadipocytes were seeded in six-well plates and RAW264.7 macrophages were maintained and passaged separately in basic media containing DMEM (Hyclone, SH30022.01B) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA) at 37°C in 5% CO_2:95% humidified air. Two days postconfluency, 3T3-L1 cells were differentiated with basic media plus 0.5 mM 3-isobutyl-1-methylxanthine, 1 mg/L INS, and 1 μM DEX for 48 h. 3T3-L1 cells were then re-fed with basic media plus 1 mg/L INS every two days to 20 days. Adipocytes which accumulated large lipid droplets were used as hypertrophied 3T3-L1 cells. Culture supernatants of each cell type with the indicated treatments were collected, respectively. Adipocytes were harvested following specific treatments.

FFA treatment

In humans, including healthy and obese people, reported plasma FFAs levels range from 0.2 to 1.4 mmol/L.[46–48] In previous cell culture-based study,[13] palmitate induced ASP resistance at high physiological FFA concentrations (0.5–1 mmol/L) comparable to total FFAs concentrations observed in obese humans. Therefore, we chose to use 0.75 mmol/L in our study. Palmitate was conjugated with BSA (palmitate–BSA [molar ratio 5:1], and control treatment with BSA was carried out in parallel). Palmitate exposure at the indicated dose for 16–18 h in co-culture system caused only minimal cell death (<5% by trypan blue exclusion).

Co-culture of adipocytes and macrophages

The hypertrophied 3T3-L1 cells were serum starved for 2 h in DMEM. The RAW264 macrophages (2.0×10^5 cells/well) were plated and grown in the top inserts containing a 0.4 μm porous membrane (Transwell, Corning, USA). The cells were co-cultured in serum-free DMEM with or without low endotoxin and lipid-free BSA (Beytotine Institute of Biotechnology, Nantong, Jiangsu, China) and then different stimuli were added sequentially to the co-cultures for the indicated times, after which both co-culture medium and adipocytes in the bottom chambers were collected.

Detection of C5L2 mRNA in 3T3-L1 adipocytes by real-time quantitative PCR analysis

For quantification of C5L2 gene expression, 3T3-L1 cells which had been exposed to the indicated treatments were homogenized in Buffer RLT (Qiagen, Shanghai, China) with β-mercaptoethanol, and RNA was isolated using Qiagen minikit spin columns as per the protocol provided by the manufacturer (Qiagen, Shanghai, China). One microgram RNA was reverse transcribed using Quantitect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, Shanghai, China). The primer sequences for C5L2 were synthesized by Sangon Biotech (Shanghai, China) according to Atefi *et al*.[49] Primers for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were ordered from Qiagen (QuantiTect Primer Assay, Shanghai, China). Specific targets were amplified using Quantitect SYBR Green PCR Kit (Qiagen, Shanghai, China) in triplicate in an Applied Biosystems 7500 machine (Applied Biosystems, Carlsbad, CA, USA) with the following parameters: uracil-*N*-glycosylase pretreatment at 50°C for 2 min; initial heat activation at 95°C for 15 min, followed by 40 cycles with denaturation at 94°C for 15 s; annealing for 30 s at 60°C for both C5L2 and GAPDH; and extension at 72°C for 30 s. Results are expressed as △△Ct ratio between target gene C5L2 and GAPDH expression.

Protein sample preparation and concentration assay

Cells were harvested using a cell scraper and homogenized in ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors (50 mmol/L Tris–Cl (pH 8.0), 150 mmol/L NaCl, 0.2 g/L NaN_3, 1 g/L SDS, 10 g/L NP-40, 100 μg/mL PMSF, 5 g/L sodium deoxycholate, 1 μg/mL aprotinin). The total cell lysates were then centrifuged at 12,000 rpm for 20 min at 4°C to remove insoluble materials. Protein concentration was determined by an Enhanced BCA Protein Assay Kit (Beytotine Institute of Biotechnology, Nantong, Jiangsu, China). Proteins were denatured by adding 5 × concentrated sodium dodecyl sulfate (SDS) reducing buffer and were boiled for 5 min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

Equal amounts of boiled extracts with 5 μL Prestained Protein Markers (Gen-View Scientific Inc., USA) were loaded in each well and separated by SDS-PAGE in a Bio-Rad Mini-Protean apparatus. Then protein extracts were transferred to a polyvinylidene fluoride membrane (Millipore, USA), blocked in 1 × Tris buffered saline containing 0.1% Tween (TBST) and 5% nonfat dry milk/3% BSA at 4°C overnight with gentle, constant agitation. The membranes were incubated with primary antibodies for 2 h at room temperature and washed twice with TBS-T buffer, followed by incubation with horseradish peroxidase-conjugated affinity-purified goat anti-rabbit secondary antibody

(1:5000, Pierce) for 2 h at room temperature with agitation. The membrane was then washed three times with TBS-T buffer. Immune complexes were detected using the Enhanced Chemiluminescence System (SuperSignal West Pico Chemiluminescent Substrate, Pierce), and band intensities were quantified using Quantity One software (Bio-rad, USA).

Measurement of cytokines

After the indicated treatments the cell culture supernatants were harvested and the production of TNFα and IL-6 measured by ELISA kits from R&D system (USA) according to the manufacturer's instructions.

Statistical analysis

All analyses were performed by two-tailed paired Student's *t*-test (GraphPad Prism 5, Graph Pad Software, Inc., San Diego, CA, USA). Values with $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered to indicate significant statistical significance.

Results

In 3T3-L1 adipocytes, α7nAChR activation normalized ASP resistance induced by co-culture with RAW264.7 macrophages supplemented with palmitate

The effects of GTS-21 on ASP-dependent expression patterns of C5L2 mRNA, Gβ protein, Gαq/11 protein, phosphorylated serine 657 PKCα (p-ser657-PKCα), and threonine 410 PKCζ (p-thr410-PKCζ) in co-cultured 3T3-L1 cells following loading with high dose of palmitate were evaluated.

As can be seen in Figure 1, addition of palmitate-BSA (molar ratio 5:1) decreased basal and ASP-stimulated C5L2 (−60.6 and −62.13%, respectively, $P < 0.05$), Gβ (−40 and −48.28%, respectively, $P < 0.05$–0.01), Gαq/11 (−42.86 and −54.55%, respectively, $P < 0.05$–0.01), p-ser657-PKCα (−36.36 and −62.61%, respectively, $P < 0.05$), and p-thr410-PKCζ (−51 and −49.77%, respectively, $P < 0.05$) in co-cultured 3T3-L1 cells. Treatment with ASP (200 nM) for 30 min had no effect on C5L2 mRNA level in co-cultured 3T3-L1 adipocytes. Treatment with BSA alone had no effect as compared to untreated control. C5L2 gene expression, G proteins, and phosphorylated PKC isoforms in ASP stimulated and control co-cultured adipocytes were indistinguishable after 16–18 h exposure to 100 μM GTS-21. There was no detectable cytotoxicity when cell viability was detected using the XTT assay under the indicated concentration. However, GTS-21 could effectively counteract the palmitate-BSA-mediated reduction in ASP-stimulated C5L2 (94.49%, $P < 0.05$), Gβ (60%, $P < 0.05$), Gαq/11 (80%, $P < 0.05$), p-ser657-PKCα (86.05%, $P < 0.05$), and p-thr410-PKCζ (42.42%, $P < 0.05$) in co-cultured adipocytes.

Therefore, GTS-21 had the capacity to prevent the palmitate-induced ASP resistance without affecting normal ASP signaling involved in TGS in co-cultured adipocytes in the trans-well system. This effect could be further reversed by treatment with selective α7nAChR blocker α-bungarotoxin (α-BGT) (Figure 2). The α7nAChR-mediated cholinergic activation could ameliorate ASP resistance in the co-culture systems.

GTS-21 has no effect on pathologic ASP resistance in 3T3-L1 adipocytes induced by indicated conditioned medium

Next, to clarify the precise mechanism of GTS-21 association with TGS, we conducted medium change experiments. First, the conditioned medium from RAW264.7 macrophages that had been treated with palmitate was added to the hypertrophied adipocyte culture. BSA and FA–BSA were removed from the macrophage cultures prior to further incubation to generate the conditioned media, and non-esterified FFAs were undetectable in such conditioned media. This FA-free conditioned media collected from RAW264.7 cells pretreated with palmitate is termed P-MCM (palmitate-macrophage-conditioned medium). As can be seen in Figure 3, 200 nM ASP treatment for 30 min had no effect on C5L2 mRNA levels in cultured adipocytes with or without addition of P-MCM. However, P-MCM treatment significantly suppressed protein expression of both basal and ASP-stimulated Gβ (−31.82 and −62.67%, respectively, $P < 0.05$–0.01), Gαq/11 (−34.33 and −33.6%, respectively, $P < 0.05$–0.01), p-ser657-PKCα (−24.8 and −24.21%, respectively, $P < 0.05$), and p-thr410-PKCζ (−24.44 and −19.04%, $P < 0.05$–0.01) in cultured adipocytes. Addition of GTS-21 did not alter protein levels of G proteins or phosphorylated PKC isoforms in ASP-stimulated and control cultured adipocytes, nor block the pathologic ASP resistance induced by P-MCM. Thus, these results indicate that components involved in ASP-dependent TGS are not targets for GTS-21. The α7nAChR-mediated cholinergic activation had no direct effect on ASP-challenged TGS signal in 3T3-L1 adipocytes.

α7nAChR activation suppresses pro-inflammatory cytokine secretion mainly from co-cultured macrophages

We previously reported that GTS-21 inhibited KC and MCP-1 secretion in isolated adipocyte cultures.[36] These chemokines induce macrophage infiltration in obese adipose tissue. The associated macrophages are thought to play a significant role in the pathogenesis of ASP resistance. Inflammation-related cytokine expression profiles in adipocytes and macrophages are distinct. We questioned whether certain cytokines in our co-cultured system were increased and whether such induction would be inhibited by GTS-21. In untreated adipocytes and macrophages, primarily TNFα secretion was detected, with little detection of IL-6 in the supernatants of both cells. Addition of 750 μM palmitate-BSA for 16–18 h provoked a 1.66-fold elevation in TNFα secretion ($P < 0.01$) and significant increases in IL-6 levels ($P < 0.01$) in 3T3-L1 cells. In RAW264.7 cells, a 2.27-fold elevation in TNFα secretion ($P < 0.01$) was detected, with greater increases in IL-6 production ($P < 0.01$) than in 3T3-L1 adipocytes. Co-incubation with ASP for 24 h further enhanced TNFα secretion ($P < 0.05$) in RAW264.7 cells but not in 3T3-L1 adipocytes, with increased IL-6 secretion ($P < 0.05$) from both cells. The conditioned media from

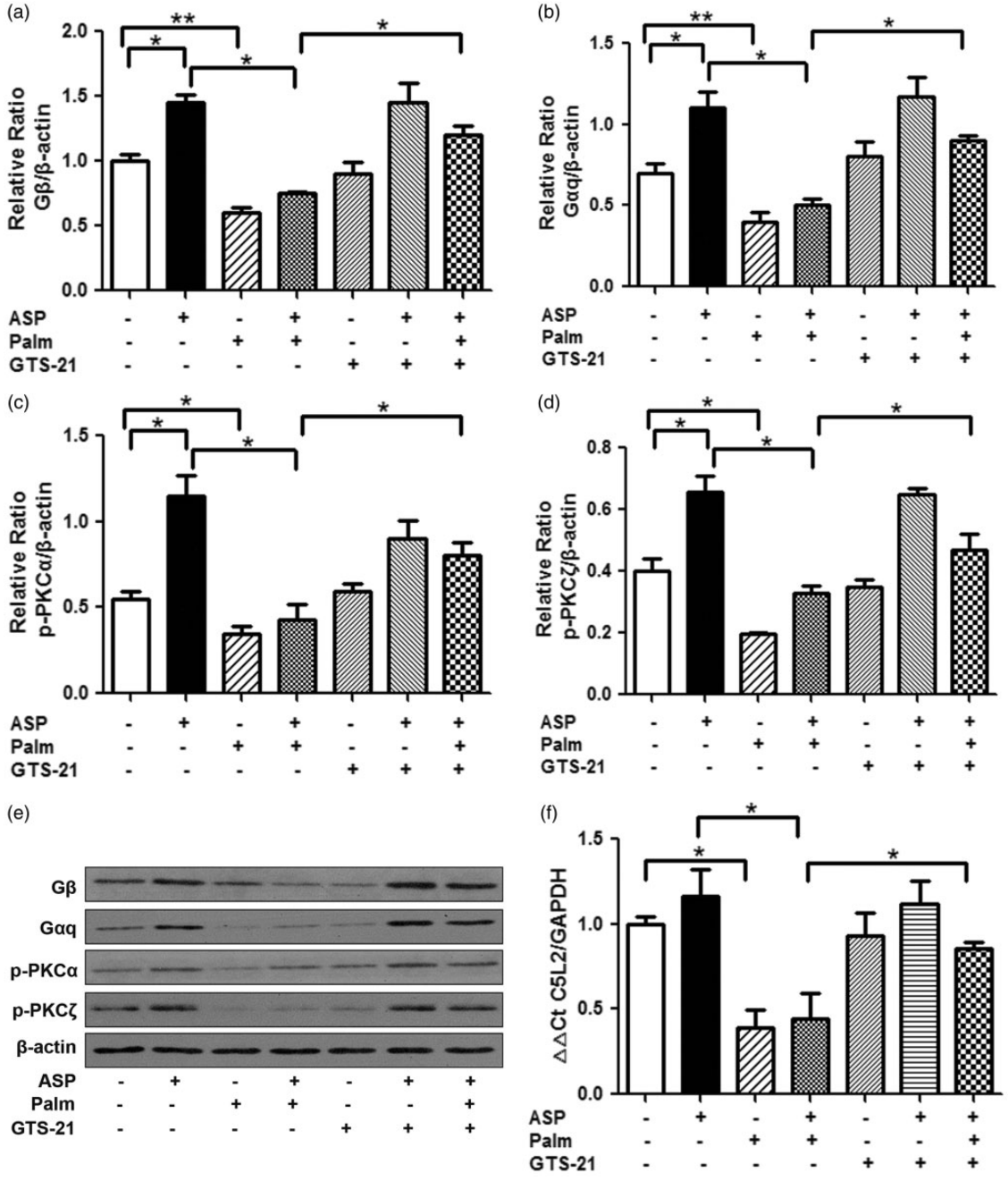

Figure 1 GTS-21 blunted the deleterious effect on ASP action in 3T3-L1 adipocytes in palmitate-stimulated co-cultured RAW264.7 macrophages. In trans-well co-culture system, cells were stimulated in the presence or absence of 750 μM palmitate (Palm) or 100 μM GTS-21 for 24 h, followed by stimulation with 200 nM ASP for 30 min as indicated. The expression of (a) Gβ, (b) Gαq or phosphorylation of PKCα (c), and PKCζ (d) were detected using specific antibodies. (e) Images shown are representative immunoblots from three independent experiments. (f) C5L2 mRNA expression in 3T3-L1 cells with the same treatment was quantified by RT-PCR. Results are expressed as means ± SEM (n = 3) where $^{*}P < 0.05$ and $^{**}P < 0.01$.
ASP: acylation-stimulating protein; PKC: protein kinase C

RAW264.7 macrophages (MCM) showed significant increases in TNFα and IL-6 secretion by 1.2- and 3.0-fold ($P < 0.05$) in 3T3-L1 adipocytes. The media from 3T3-L1 adipocytes (adipocyte-conditioned medium [ACM]) also induced TNFα and IL-6 secretion by 1.48-fold ($P < 0.01$) and 3.64-fold ($P < 0.05$) in RAW264.7 cells. When co-incubating with ASP and palmitate for 24 h, the co-culture system resulted in exacerbated productions of these pro-inflammatory cytokines as compared to single culture (Figures 4 and 5), but co-addition of ASP for just 30 min did not alter IL-6 and TNFα secretion. These increases were significantly suppressed when co-incubated with GTS-21. It is worth noting that both TNFα and IL-6 are mainly derived from macrophages in the co-cultured system as can be seen in Figures 4 and 5. The α-BGT effectively reversed the anti-inflammatory potential of GTS-21 with a rebound of TNFα and IL-6 levels. Combined with previous results, α7nAChR-mediated cholinergic activation

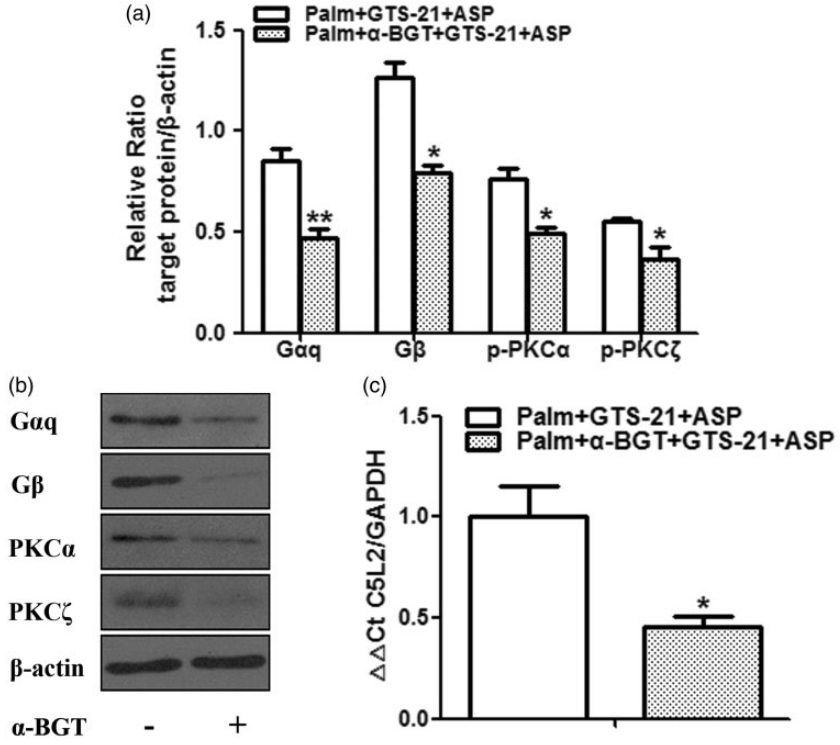

Figure 2 α-BGT diminished the mitigating effect of GTS-21 on ASP signaling in palmitate-stimulated co-cultured RAW264.7 macrophages. In trans-well co-culture system, cells were pretreated or not with α-BGT (2 μM) in the presence or absence of 750 μM palmitate (Palm) plus 100 μM GTS-21 for 24 h, followed by stimulation with 200 nM ASP for 30 min as indicated. (a) The expression of Gβ, Gαq, or phosphorylation of PKCα and PKCζ were detected using specific antibodies. (b) Images shown are representative immunoblots from three independent experiments. (c) C5L2 mRNA expression in 3T3-L1 cells with the same treatment was quantified by RT-PCR. Results were expressed as means ± SEM (n = 3), where $^{*}P < 0.05$ and $^{**}P < 0.01$.
ASP: acylation-stimulating protein; α-BGT: α-bungarotoxin; PKC: protein kinase C; RT-PCR: reverse transcription polymerase chain reaction

not only inhibited chemokine levels in adipocytes to indirectly decrease infiltration of macrophages,[36] but also restricted pro-inflammatory cytokine secretion in co-cultured cells.

GTS-21 has no effect on pathologic ASP resistance in 3T3-L1 adipocytes induced by TNFα

Previous study[15] has shown that TNFα significantly decreased C5L2 expression and ASP binding in both 3T3-L1 preadipocytes and adipocytes. The changes in C5L2 mRNA tend to begin at lower concentrations (0.1 ng/mL) than changes in cell surface C5L2 protein levels in preadipocytes. For adipocytes, both mRNA and ASP binding changes occur at higher concentrations (100 ng/mL). Whether TNFα could directly affect the intracellular targets of ASP-stimulated TGS pathway is not known. Thus, we have examined the effect of TNFα on protein expression of ASP-stimulated G proteins and phosphorylated PKC isoforms in adipocytes. As can be seen in Figure 6, 100 ng/mL TNFα pretreatment for 18 h resulted in a significant decrease in ASP-induced Gβ (−68%, $P < 0.05$), Gαq/11 (−52%, $P < 0.01$), p-ser657-PKCα (−37.79%, $P < 0.05$), and p-thr410-PKCζ (−57.39%, $P < 0.05$). No obvious change was observed in ASP-stimulated expression of C5L2, G proteins, and phosphorylated PKC isoforms when treated with IL-6 at the indicated concentrations. TNFα but not IL-6 could induce ASP resistance in 3T3-L1 adipocytes. GTS-21 had no significant effect on TNFα-induced signal impairment in ASP-related TGS.

Discussion

To date, ASP has been identified as serving a dual role in adipocytes. In the inflamed obese adipose tissue, ASP is involved in initiating inflammatory reactions, while its ability to stimulate TGS is weakened due to ASP resistance. This may be explained through two different possibilities: (1) an initial accumulation of TGs stimulated by ASP contributes to inflammatory response; inflammatory factors subsequently impair the ASP sensitivity preventing further lipid load in larger, hypertrophic adipocytes; (2) a direct inflammatory state induced by ASP interferes with the fat storage stimulation of ASP. With either or both possibilities, the subsequent ASP-resistant state could be recognized as a self-protective mechanism.

Adipocytes serve both as a depot for energy storage and as an endocrine gland secreting adipokines that contribute to energy homeostasis, inflammation, and the innate immune response. It can be physiologically beneficial to integrate these two functions in a single cell type. Previous studies have found that adipocytes exert a strong pro-inflammatory effect on macrophages. In obese adipose tissue, remodeling and paracrine interaction of

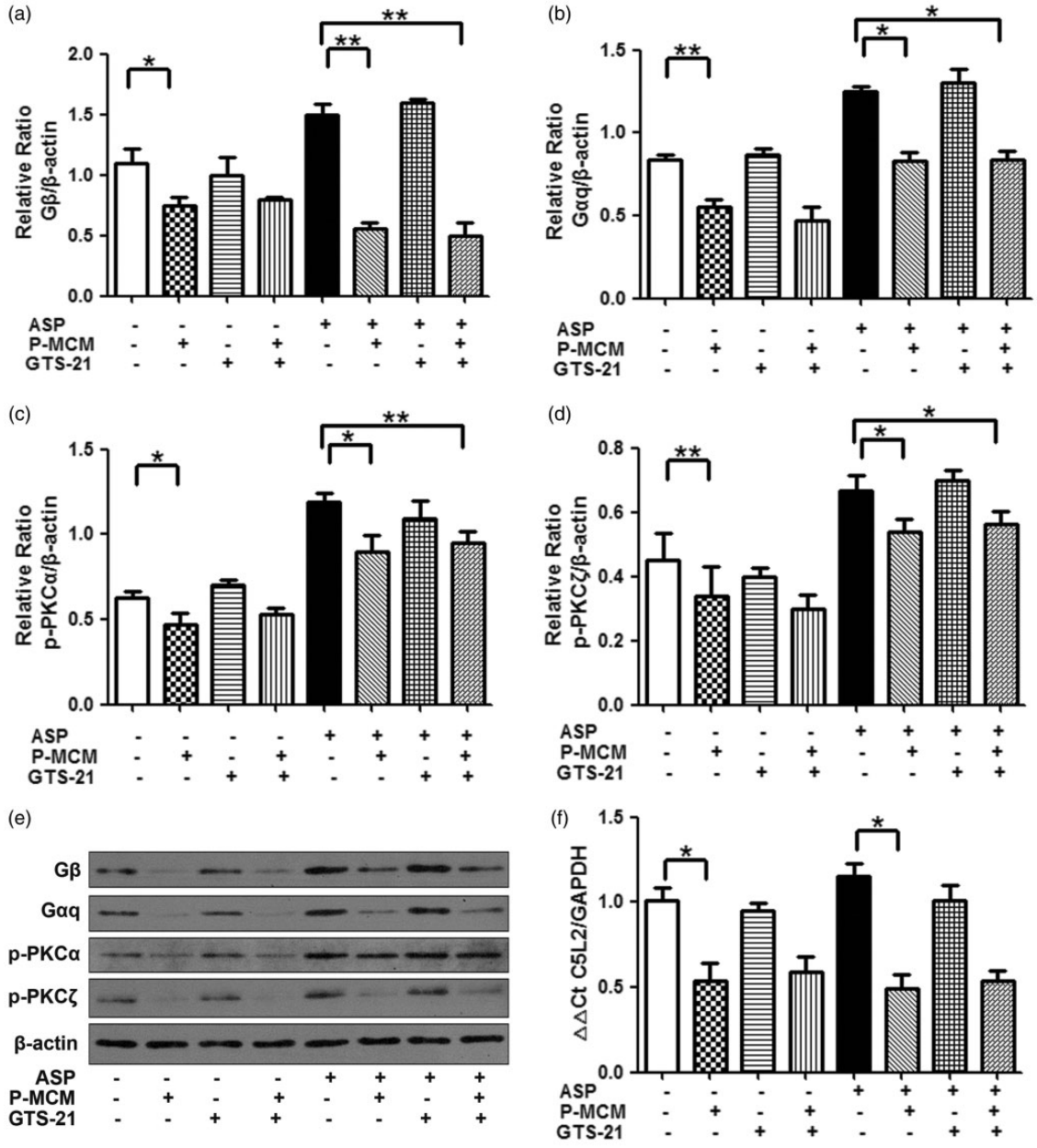

Figure 3 GTS-21 did not ameliorate the ASP signaling impairment in 3T3-L1 adipocytes caused by P-MCM. 3T3-L1 adipocytes were untreated, treated overnight (10 h) with P-MCM alone, or treated with GTS-21 (100 μM) before P-MCM for 1 h. Following 2 h of serum starvation, adipocytes were stimulated or not with ASP (200 nM for 30 min) and then assayed for expression of (a) Gβ, (b) Gαq or phosphorylation of PKCα (c), and PKCζ (d). (e) Images shown are representative immunoblots from three independent experiments. (f) C5L2 mRNA expression in 3T3-L1 cells with the same treatment was quantified by RT-PCR. Results were expressed as means ± SEM (n = 3) where $^{*}P < 0.05$ and $^{**}P < 0.01$.

ASP: acylation-stimulating protein; PKC: protein kinase C; P-MCM: palmitate-macrophage-conditioned medium; P-MCM: palmitate-macrophage-conditioned medium; RT-PCR: reverse transcription polymerase chain reaction

macrophages and adipocytes contribute to low-grade chronic inflammation. *In vitro* studies showed that direct co-culture of adipocytes and macrophages leads to synergistic effects on secretion of C3 and production of ASP in adipocytes.[19] Adipocytes usually exhibit higher lipolytic activity in co-cultured inflammation-inducing conditions.[29,50] FFAs, which are released from adipocytes, exert activity similar to that of LPS from bacterial cytoderm, inducing inflammatory changes in macrophages. Elevated circulating FFAs in obesity also potentially contribute to the development of ASP resistance and INS resistance. Palmitate, for instance, is the most abundant FFA released from dysregulated adipocytes.[51] Palmitate at levels up to 0.5–1 mM can induce ASP resistance in mature adipocytes.[14] Strikingly, our results further suggest that conditioned media from palmitate-challenged macrophages also induce an ASP-resistant phenotype in adipocytes. The effect is further additive to those of co-culture systems with high FFA load (in the presence of palmitate). These data suggest that the interplay between adipocytes and macrophages is a possible mechanism for adipose tissue ASP resistance. The production of certain cytokines

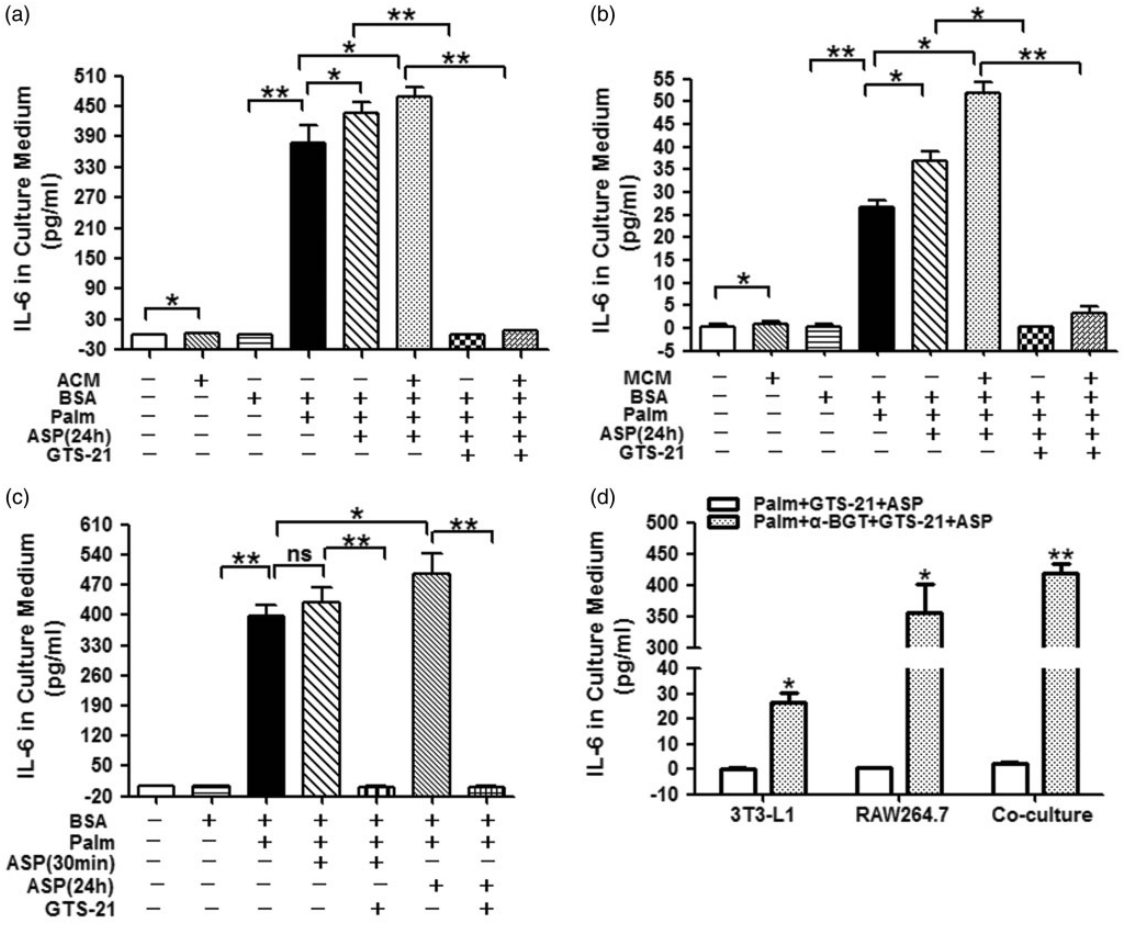

Figure 4 Role of α7nAChR selective activation suppresses excessive IL-6 secretion by 3T3-L1 adipocytes, RAW264.7 macrophages, and co-culture with indicated treatments. (a) RAW264.7 macrophages were treated with ACM, BSA, 750 μM palmitate (BSA complexed), 750 μM palmitate (BSA complexed) plus 200 nM ASP or 10-4M GTS-21 30 min before 750 μM palmitate (BSA complexed) plus 200 nM ASP for 24 h. (b) The hypertrophied 3T3-L1 adipocytes were treated with MCM, BSA, 750 μM palmitate (BSA complexed), 750 μM palmitate (BSA complexed) plus 200 nM ASP or 10-4M GTS-21 30 min before 750 μM palmitate (BSA complexed) plus 200 nM ASP for 24 h. (c) The trans-well co-culture system was treated for 24 h with ACM, BSA, 750 μM palmitate (BSA complexed), 750 μM palmitate (BSA complexed) plus 200 nM ASP (30 min or 24 h, respectively) or 10-4 M GTS-21 30 min before 750 μM palmitate (BSA complexed) plus 200 nM ASP (30 min or 24 h, respectively). (d) The single culture or co-culture systems were combined with or without 2 μM α-BGT for 30 min. The supernatants were evaluated by ELISA. Results were expressed as means ± SEM (n = 3), where $^{*}P < 0.05$ and $^{**}P < 0.01$.
ASP: acylation-stimulating protein; α-BGT: α-bungarotoxin; BSA: bovine serum albumin; ELISA: enzyme-linked immunosorbent assay; MCM: macrophage-conditioned medium

affecting immune cell responses through the cross talk between these two cells during high fat diet or dyslipidemia is earlier than the development of direct lipid-induced ASP resistance.

Palmitate has been shown to be more potent in inducing inflammation than lipopolysaccharide (LPS). High concentrations of palmitate induce increases in TNFα and IL-6 secretion from adipocytes, although not to the same extent as that typically elicited from macrophages in our study. TNFα secreted from palmitate-challenged adipocytes may be insufficient to induce ASP resistance, because the production levels are very small or negligible compared with that of macrophages. Other studies have even suggested that the release of TNFα into the culture medium was actually attenuated by palmitate in a dose-dependent manner, at least within the time line of the experiments conducted.[52] Indeed, the elevated TNFα and IL-6 seem to be independent of the source of the adipocytes and macrophages, because similar results were obtained when different macrophage cell lines or primary macrophages were treated with ACM derived from different adipose tissue depots or co-cultured with an adipocyte line regardless of any other additional treatment, mimicking the inflammatory microenvironment in obese adipose tissue.[29,51–54] Ebke *et al.* even found that macrophages but not adipocytes are the source of TNFα expression in obese adipose tissue.[55] It should be noted that *in vivo* there is an ongoing dynamic of adipocyte-to-macrophage ratio, due to abundant apoptosis of adipocytes and continuous aggregation of recruited macrophages in obese adipose tissue. Therefore, a limitation of the present co-culture experiments is the maintenance of a fixed 1:1 adipocyte-to-macrophage ratio, which may not accurately reflect the high fat-induced inflammatory microenvironment. Additionally, we detected that 24 h incubation of ASP potentially increased the palmitate effect on IL-6 secretion from both cells and TNFα from RAW264.7 cells. The secretion of TNFα and IL-6 increased more in co-culture in combination with ASP than the single

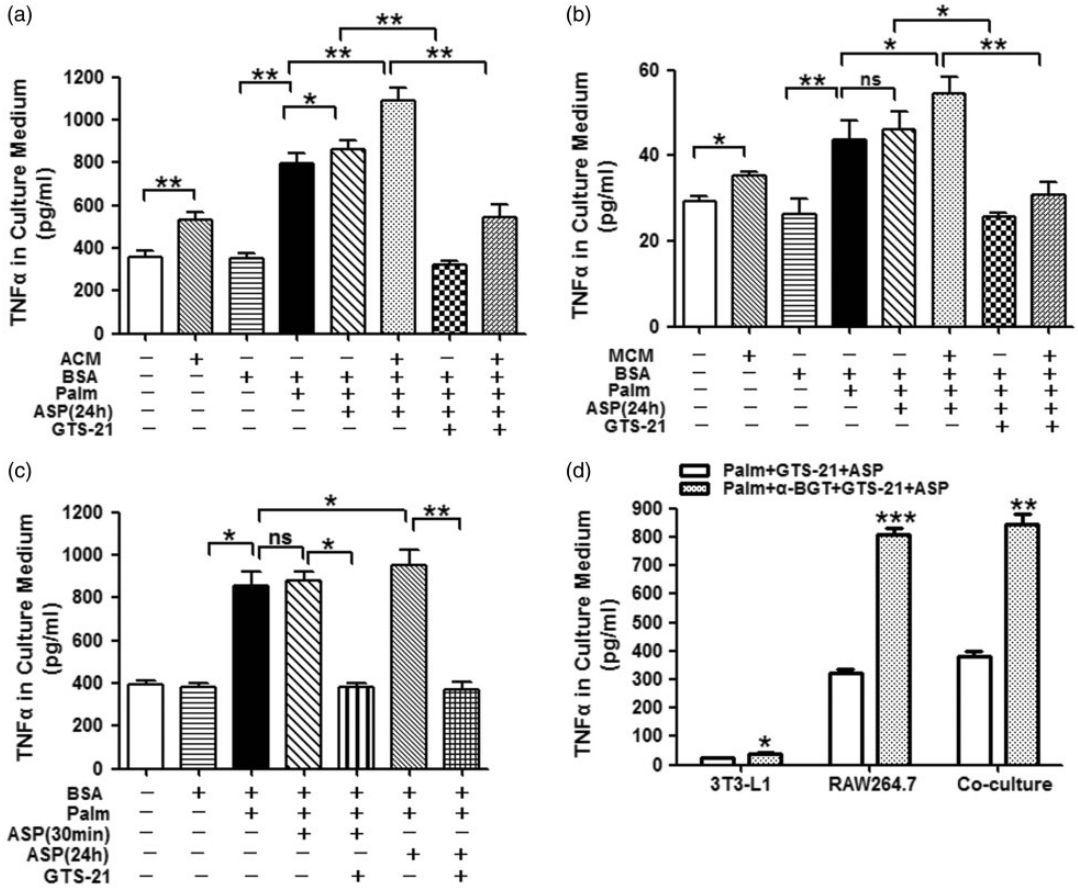

Figure 5 Role of α7nAChR selective activation suppresses excessive TNFα secretion by 3T3-L1 adipocytes, RAW264.7 macrophages and co-culture with indicated treatments. (a) RAW264.7 macrophages were treated with ACM, BSA, 750 μM palmitate (BSA complexed), 750 μM palmitate (BSA complexed) plus 200 nM ASP or 10-4M GTS-21 30 min before 750 μM palmitate (BSA complexed) plus 200 nM ASP for 24 h. (b) The hypertrophied 3T3-L1 adipocytes were treated with MCM, BSA, 750 μM palmitate (BSA complexed), 750 μM palmitate (BSA complexed) plus 200 nM ASP or 10-4 M GTS-21 30 min before 750 μM palmitate (BSA complexed) plus 200 nM ASP for 24 h. (c) The trans-well co-culture system was treated for 24 h with ACM, BSA, 750 μM palmitate (BSA complexed), 750 μM palmitate (BSA complexed) plus 200 nM ASP (30 min or 24 h, respectively) or 10-4M GTS-21 30 min before 750 μM palmitate (BSA complexed) plus 200 nM ASP (30 min or 24 h, respectively). (d) The single culture or co-culture systems were combined with or without 2 μM α-BGT for 30 min. The supernatants were evaluated by ELISA. Results were expressed as means ± SEM (n = 3), where $^{*}P < 0.05$ and $^{**}P < 0.01$.

ASP: acylation-stimulating protein. ASP: acylation-stimulating protein; α-BGT: α-bungarotoxin; BSA: bovine serum albumin; ELISA: enzyme-linked immunosorbent assay; MCM: macrophage-conditioned medium; TNFα: tumor necrosis factor alpha

culture systems. Overall, high doses of palmitate induced adipocyte inflammatory programs and a pro-inflammatory phenotype in macrophage. Conditioned media from both challenged cells contributed to their bidirectional cross talk. We can reasonably infer that long-term supplementation with ASP would enhance the pro-inflammatory effect of palmitate. The consequence would be an increase in the potential ASP resistance. *In vivo*, acute administration of ASP in DIO mice increased plasma levels of IL-6 and TNFα and elevated their mRNA contents in liver and adipose tissue. However, chronic supplementation of ASP induced increased IL-6 mRNA content only in adipose tissue but had no effect on TNFα expression and secretion. Reconstitution of C3−/− mice with high-dose ASP immediately following partial hepatectomy resulted in increased serum IL-6 and TNFα levels 6 h later.[56] Thus, it can be seen that the pro-inflammatory action of ASP is tissue specific and environment specific.

In this study, we observed direct interference of ASP signal mediators for TGS in adipocytes by TNFα. This lends even more support to the importance of inflammatory factors in the development of ASP resistance since previous work has shown that TNFα may potentially decrease ASP sensitivity.[15,19] TNFα has a direct lipolytic activity and promotes self-secretion, altogether aggravating ASP resistance. TNFα is a typical pro-inflammatory cytokine that has been implicated in tissue pathology associated with lipid overload.[57] Adipose tissue expression of TNFα is increased in a variety of experimental models of obesity and in obese humans[30–32,37] and may represent an important link between obesity and ASP resistance. However, we cannot entirely exclude the possibility that some other inflammatory mediators are affecting the ASP-induced TGS signal.

The protein levels of α7nAChR in human adipocytes are substantially decreased in obese individuals.[33] Weight loss partially restores its expression.[33] Further, decreased vagus nerve activity and expression of other cholinergic components such as acetylcholine esterase BChE in diabetic and obese mice raise the intriguing possibility that the dysfunction of cholinergic signaling in adipose tissue may

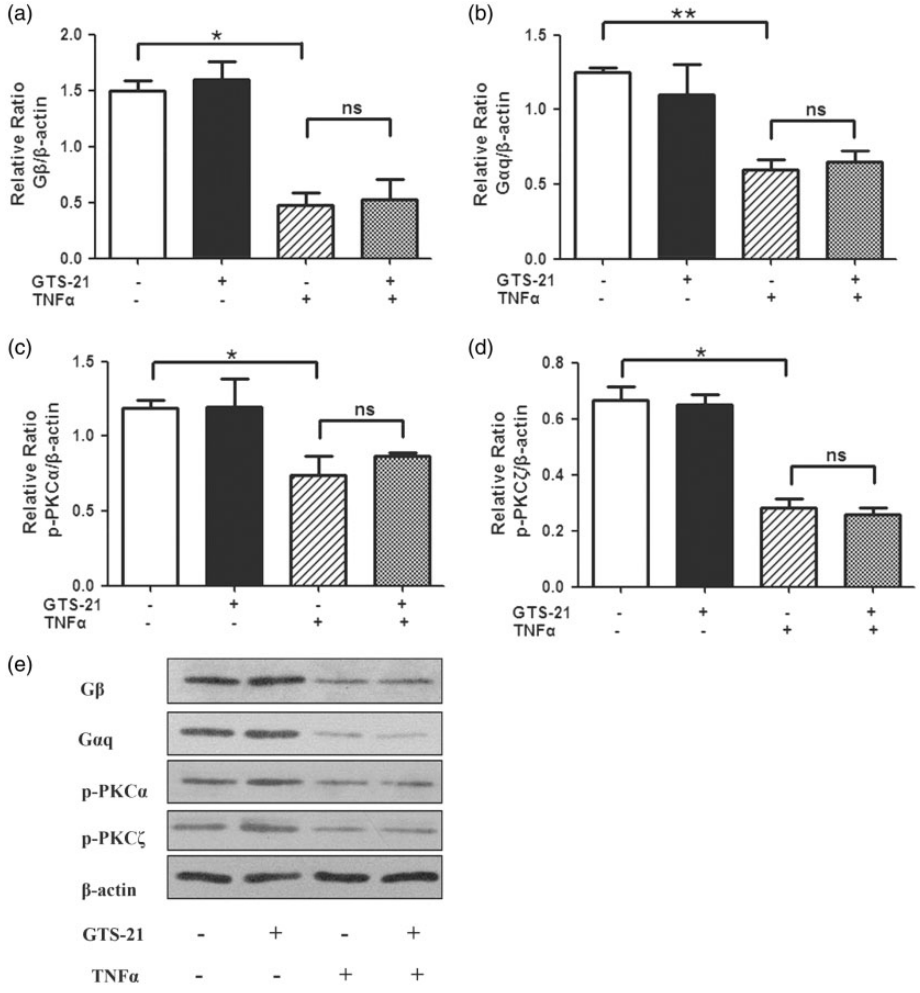

Figure 6 GTS-21 did not reverse decreased expression of Gβ, Gαq, or phosphorylation of PKCα and PKCζ in 3T3-L1 adipocytes induced by TNFα. The cells were untreated, treated overnight (18 h) with TNFα (100 ng/mL) alone, or treated with GTS-21 (100 μM) before TNFα for 1 h. Following 2 h of serum starvation, adipocytes were stimulated or not with ASP (200 nM for 30 min) and then assayed for expression of (a) Gβ, (b) Gαq or phosphorylation of PKCα (c) and PKCζ (d). (e) Images shown are representative immunoblots from three independent experiments. Results were expressed as means ± SEM (n = 3), where $^{*}P < 0.05$ and $^{**}P < 0.01$.
ASP: acylation-stimulating protein; PKC: protein kinase C; TNFα: tumor necrosis factor alpha

contribute to the pathogenesis of obesity and its related comorbidities.[35] Therefore, targeting cholinergic mechanisms may provide novel therapeutic approaches in the treatment of obesity-related disorders. Administration of the selective α7nAChR agonist TC-7020 to lepr db/db mice significantly suppressed serum TNFα levels and alleviated metabolic parameters including decreased levels of plasma TGs, HbA1c, and glucose.[31] All these effects are abolished by co-administration of the selective α7nAChR antagonist methyllycaconitine.[31] However, treatment with agonist selectively targeting α4/β2 nAChR failed to achieve the same effect.[34] This indicates that α7nAChR is unique in the cholinergic system, having been specifically linked to controlling obesity-related metabolic disorders through suppression of inflammation. Both macrophages and adipocytes in adipose tissue possess functional α7nAChR.[32,33] We have previously found that α7nAChR attenuated ASP-initiated inflammation by disturbing post-translational modification in adipocytes.[36] Our present study demonstrated that α7nAChR activation does not ameliorate ASP resistance induced by either macrophage-conditioned medium or TNFα in a single culture system. However, several lines support that α7nAChR possesses a suppressive effect against LPS or stearic acid-stimulated expression of pro-inflammatory cytokines including TNFα in obese human and rodents.[32] Systemic reduction of TNFα levels by choline in endotoxin-treated mice was eliminated in α7nAChR knockout mice during endotoxemia. Similarly, vagus nerve stimulation does not inhibit TNFα release in α7nAChR knockout mice. In co-culture, α7nAChR selective agonist exerts similar effects to a TNFα neutralizing antibody. In other words, TNFα may be an important mediator connecting the cholinergic pathway and ASP-stimulated TGS metabolic processes. It might be speculated that α7nAChR indirectly sensitizes the ASP effect on TGS through downregulation of TNFα. In addition, α7nAChR may affect lipolytic rates and plasma FFA levels,[31] then halting the vicious cycle between FFA and TNFα to a certain extent. Further, nicotine may indirectly influence the rate of C3 conversion to ASP through altering expression of adipokine profiles, although there exists discrepancies in cell lines from different species.[58,59] This discussion does not

exclude that paracrine signals other than TNFα might be involved in mediating cholinergic regulation of ASP-stimulated TGS in co-culture. Nicotine regulates expression of certain adipokines in adipocytes in the presence of co-cultured macrophages but not in adipocyte single culture, although evidence is still lacking to support that the response is mediated by α7nAChR. It is tempting to speculate that α7nAChR dysfunction interferes with the communication system between adipocytes and macrophages, which may contribute to inflammatory changes and metabolic imbalance within obese adipose tissue. Additional studies are needed to elucidate the pathophysiologic significance of this receptor in intracellular cross talk *in vivo* and the underlying molecular mechanism in adipose tissue.

In summary, the cholinergic regulation of ASP effects is more intricate than initially proposed. On the one hand, the α7nAChR-mediated pathway enhances ASP efficiency to decrease TG/FA substrate cycling and enhance TG trapping in adipose tissue, allowing for reduced energy expenditure. On the other hand, α7nAChR directly inhibits the inflammatory reaction initiated by ASP.

Authors' contributions: Wu J and Jiao ZY contributed equally to this paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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