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Regular paper

Preventive effect of salicin ether against type-2 diabetes mellitus through targeting PPARγ-regulated gene expression

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Diabetes mellitus is the syndrome associated with metabolism having complicated pathogenesis and its morbidity rate is rapidly increasing every year. The present study investigated the preventive effect of salicin ether against type-2 diabetes and explored the underlying mechanism. Salicin ether reduced PPARγ-LBD level and transcriptional property of RXRα-PPARγ in 293T cells. The rosiglitazone significantly (p<0.01) increased grease droplet accumulation in adipocytes in comparison to control adipocytes. Increased grease droplet accumulation by rosiglitazone in adipocytes was reversed on treatment with salicin ether in dose-dependent manner. Salicin ether treatment of the adipocytes effectively suppressed rosiglitazone induced expression of FAS, C/EBPα, aP2, and HMG-CoA genes. Treatment of the adipocytes with salicin ether led to a prominent decrease in rosiglitazone mediated increase in aP2, CHIP, and C/EBPα protein expression. The inhibitory effect of rosiglitazone on expression of p-Akt/t-Akt, PPARa, p-FoxO1/t-FoxO1, and p-AMPK/t-AMPK was significantly (p<0.01) alleviated in the adipocytes by salicin ether. In summary, the present study demonstrated that salicin ether suppressed PPARγ activity and adipocyte differentiation. Moreover, the activation of FoxO1/Akt/AMPK was up-regulated and FAS/EBPα/aP2/HMG-CoA level inhibited by salicin ether in the adipocytes. Thus, salicin ether may be studied further for possible role in the treatment of diabetes.

Keywords: salicin ether, condensation reaction, adipocytes, type-2 diabetes, glucolipids

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Abbreviations: PPARγ, peroxisome proliferator-activated receptor-γ; RXRα, retinoid X receptor-α

INTRODUCTION

Diabetes mellitus is a metabolic syndrome associated with complicated pathogenesis and its rate of morbidity has been increasing every year. The syndrome is characterized by chronic hyperglycemia because of the loss of beta-cells in pancreas leading to deficiency of insulin (Lin *et al.*, 2018; Wang *et al.*, 2015). Despite advanced medical technology, no effective treatment free from side effects has been developed for diabetes mellitus till date (Samuel & Shulman, 2012). Excessive increase in sugar level and elevated rate of lipid metabolism due to insulin deficiency or high glucagon level are the main factors involved in type-2 diabetes mellitus development (Kahn *et al.*, 2006). Clinicians have found that diabetes mellitus patients suffer from hyperglycemia as well as hyperlipemia (Kahn *et al.*, 2006). Diabetes mellitus is considered to be a developing health problem globally and a burden on the societies because it largely affects life standard of the people. It has been recommended by the American and European Diabetes Association that the level of glycosylated hemoglobin in 7% of the patients with diabetes should be studied to identify undesired glucose in blood (Kumar *et al.*, 2010). The orally taken anti-diabetic compounds are associated with many harmful effects while as insulin administration leads to increased body weight and hypoglycemia. This emphasizes need to develop efficient and novel treatment strategy for diabetes mellitus type 2 urgently (Halperin & Goldfine, 2013).

The metabolism of glucolipids in body is regulated by peroxisome proliferator-activated receptor-γ (PPARγ) (Houseknecht *et al.*, 2002). The PPARγ belongs to the family of nuclear receptors and is dependent on ligands for its activity (Bajaj *et al.*, 2007). The receptor on ligation followed by activation combines with retinoid X receptor-α (RXRα) to afford heterodimer (Sharma & Staels, 2007). The expression of various genes on response element (PPRE) is regulated by the heterodimer (Sharma & Staels, 2007). The thiazolidinedione class of chemotherapeutics, like rosiglitazone used clinically for diabetes mellitus type 2 treatment, decrease hypoglycemia by acting as agonists for PPARγ (Shearer & Billin, 2007). The clinical use of these chemotherapeutics has led to cardiovascular disorders and increased body weight (Shearer & Billin, 2007). Recently anti-diabetic studies have focused on knocking out of PPARγ gene or targeting its expression at an intermediate stage to eliminate resistance of insulin to fatty foods for development of effective strategy (Clement *et al.*, 2000; Kadowaki, 2001). The use of PPARγ antagonists for diabetes mellitus has no side effects and is therefore preferred over previous treatments (Zinman, 2001; Wojtowicz *et al.*, 2014; de Boer *et al.*, 2010).

Salicin, a natural product extracted from the bark of White Willow, has been found to possess analgesic and anti-inflammatory properties. Pharmacodynamics has revealed that salicylic acid formed from the salicin within the body is actually responsible for anti-inflammatory and pain-relieving properties (Pilotto *et al.*, 2004). It also inhibits COX-2 activity, like that of aspirin, but has no anticoagulant potential (Hawkey, 2004). Salicin isolated from the extract of white willow bark has been found to be effective in treating knee and/or hip osteoarthritis pain (Bigler *et al.*, 2001) as well as back pain (Macarthur *et al.*, 2005). In the present study antagonistic effect of salicin ether (Fig. 1) for PPARγ was investigated with possible implications in anti-diabetic treatment.

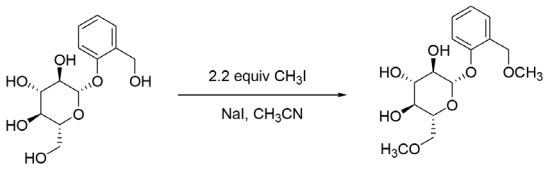

Figure 1. Preparation of Salicin ether.

MATERIALS AND METHODS

Cell lines and culture

The preadipocyte, 3T3-L1, and 293T cells were obtained from the American Type Culture Collection, Manassas, VA, USA. Maintenance of the cells was made in DMEM which contained fetal bovine serum (10%) as well as antibiotics (penicillin-streptomycin). For 24 h both the cell lines were cultured under an atmosphere of 5% CO_2 at 37ºC.

Synthesis of salicin dimethyl ether

To a solution of salicin (1000 mg) in acetonitrile solvent was added methyl iodide (2 equivalents) and sodium hydride (1.2 equivalent) as base. The reaction mixture was stirred for 24 h till completion as monitored by thin layer chromatography. The reaction mixture was charged on silica gel column through which a mixture of solvents (hexane and dichloromethane) in 90:10 ratio was passed to purify the product. TLC was performed in 98:2 hexane and dichloromethane solvent mixture. The product formed was characterized by ^{1}H NMR, ^{13}C NMR, and HRMS techniques.

Determination of luciferase activity

The effect of Salicin ether on rosiglitazone mediated changes in RXRα-PPARγ transcription and expression of PPARγ was analyzed by luciferase activity assay. The 293T cells (2×10^5) were dispersed in 24-well plates in DMEM until reaching 70–80% confluence. The plasmids for UAS-TK-Luc and PPARγ-LBD were transfected in the cells by employing Ca/PO_4 Transfection kit. The cells following 8 h of plasmid transfection were transferred in medium mixed with 2, 4, 8 and 16 μM Salicin ether. Following 18 h, the medium was decanted, and PBS was used for plate washing twice. The lysis of cells was carried out on adding lysis buffer 130 μl followed by 25 min incubation. The luciferase activity was assessed using luciferase kits available commercially (Dual Luciferase®; Promega Corporation). The fluorescein was used as control.

Analysis of differentiation in 3T3-L1 pre-adipocyte

The effect of Salicin ether and rosiglitazone on preadipocyte differentiation was analyzed by known methodology (Chawla *et al*., 1994). The 3T3-L1 cells were put in six-well plates at 2×10^5 cells per well concentration and maintained until reaching 100% confluence. The cells were transferred in medium which contained mixture of MIX (0.2 mg/l), dexamethasone (0.4 mg/l), and insulin (0.9) mg/l on the 4th day. Following 3-day culture, medium was exchanged by new medium containing insulin (0.9) mg/l and incubation for 3 more days was continued. The adipocytes after differentiation were cultured in DMEM plus 10% FBS for 48 h. The adipocytes were exposed to 2, 4, 8, and 16 μM Salicin ether, rosiglitazone or only DMSO (control) for 6 days. The PBS washing of cells was done following removal of differentiation medium. The accumulation of fats in adipocytes was assessed on staining with Oil Red O using commercial kits. The BX 50 microscope (Olympus Corporation, Tokyo, Japan) was used to observe the cells for differentiation.

Quantitative polymerase chain reaction (PCR)

The 3T3-L1 adipocytes at 2×10^5 cells per well concentration were suspended in 6-well plates after differentiation. The adipocytes were exposed to 2, 4, 8, and 16 μM Salicin ether, rosiglitazone or only DMSO (control) for 24 h. The total RNA extraction from adipocytes was carried out on treatment with TRIzol reagent (Invitrogen). The 20 μg samples were subjected to cDNA synthesis using PrimeScriptTM RT kit (Takara Biotechnology, Co., Ltd., Dalian, China) by reverse transcription. The Applied Biosystems 7300-instrument was used for amplification of the cDNA solution consisting of total RNA (100 ng) according to manual instructions. The reaction mixture used consisted of forward and backward primers (200 nM), targeted cDNA specific probes (100 nM), and Universal Master mix (TaqMan). ABI Prism 7700 Sequence Detector was used for conducting PCR reaction using the conditions: 52°C for 1 min, 93ºC for 4 min, then 42 cycles of 93ºC for 10 sec and 58ºC for 1 min. The Detection software (PE Biosystems Japan) was used for examination of the data.

Western blot analysis

The adipocytes following differentiation were rinsed in ice-cold PBS and subsequently lysed in TNN buffer [consisting of Triton X-100 (1.2%), Nonidet P-40 (1.2%), along with inhibitors for phosphatases and proteases, $PhCH_2SO_2F$, sodium fluoride (110 mM), sodium vanadate (450 mM), and sodium phosphate (450 mg/ml)]. The cells were transferred to eppendorf tubes, centrifuged at 4ºC for 15 min at 12000×*g*. The BCA kit was employed for analysis of protein concentration. The samples of 10 μg were put into wells which contained 8–10% SDS-polyacrylamide gel and resolved by electrophoresis. Transfer of proteins to PVDF membrane was followed by blocking at room temperature with PBS containing Tween-20 (0.05%) and dry-milk (5%) for 1.2 h. Incubation was carried out overnight at 4ºC with aP2, CHIP, p-Akt, t-Akt, PPARa, p-FoxO1, t-FoxO1, p-AMPK, t-AMPK, and C/EBPα primary antibodies (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following, membrane washing incubation at room temperature was done with HRP-conjugated goat anti-rabbit. Visualization and quantification were made using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc., USA) on Super RX-N film and Image J software, respectively.

Statistical analysis

The data expressed are mean ±S.D. The student's *t*-test and ANOVA followed by Bonferroni's post *hoc test* were used for comparison of data obtained. The data was analyzed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The values were taken significant at $p<0.05$.

RESULTS

Salicin ether inhibits PPARγ activity

Salicin ether exhibited inhibitory effect on PPARγ-LBD activity in 293T cells in dose-based manner

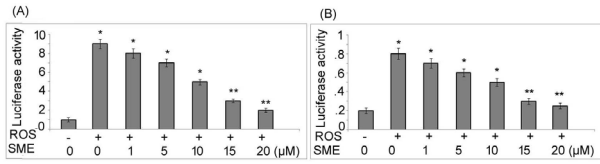

Figure 2. Effect of salicin ether on (A) PPARγ-LBD activity and (B) PPRE-Luc transcription in 293T cells.
Transfection of the cells with plasmids for PPARγ-LBD, UAS-TK-Luc and pRL-SV40 (reference) was followed by treatment with salicin ether after 6 h. Luciferase activity kit was used for determination of luciferase activity in the cells after 24 h of salicin ether treatment. *p<0.01 and **p<0.001 *vs.* DMSO group

(Fig. 2A). Rosiglitazone exposure significantly (p<0.01) elevated PPARγ-LBD level in 293T cells but salicin ether treatment reversed the effect. In rosiglitazone exposed 293T cells, transcriptional potential of RXRα-PPARγ showed a prominent increase compared to the control (Fig. 2B). However, salicin ether treatment reversed the rosiglitazone induced increase in transcriptional potential of RXRα-PPARγ in dose-dependent manner.

Salicin ether suppresses differentiation of 3T3-L1 preadipocytes

Differentiation of 3T3-L1 pre-adipocytes was measured after exposure to rosiglitazone alone or combination of salicin ether and rosiglitazone using Oil Red O staining assay (Fig. 3). Exposure to rosiglitazone caused a significant (p<0.05) increase in grease droplet accumulation in adipocytes compared to the control adipocytes. The increased grease droplet accumulation by rosiglitazone in adipocytes was reversed on treatment with salicin ether. Reversal of rosiglitazone induced increase in grease droplet accumulation in adipocytes by salicin ether was found to be dose-dependent. Rosiglitazone induced increase in grease droplet accumulation was completely prevented by salicin ether treatment at 20 μM concentration.

Salicin ether inhibits lipid metabolism related gene expression

In salicin ether treated adipocytes, lipid metabolism related gene expression was determined using RT-PCR (Fig. 4). Administration of rosiglitazone to adipocytes led to a significant (p<0.01) increase in expression of FAS and HMG-CoA genes compared to the control. Additionally, rosiglitazone exposure also elevated C/EBPα and aP2 expression in adipocytes in comparison to the control. However, treatment of adipocytes with salicin ether suppressed rosiglitazone mediated increase in expression of FAS, C/EBPα, aP2, and HMG-CoA genes in dose-based manner.

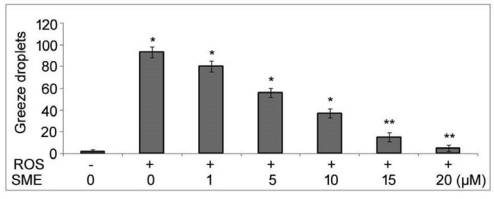

Figure 3. Effect of salicin ether on adipocyte differentiation.
The adipocytes were treated with DMSO (control), rosiglitazone and various concentrations of (1, 5, 10, 15, and 20 μM) salicin ether. Adipocyte differentiation was measured after 24 h of salicin ether treatment using Oil Red O staining assay by BX 50 microscope (Olympus Corporation, Tokyo, Japan). *p<0.05 and **p<0.01 *vs.* control group.

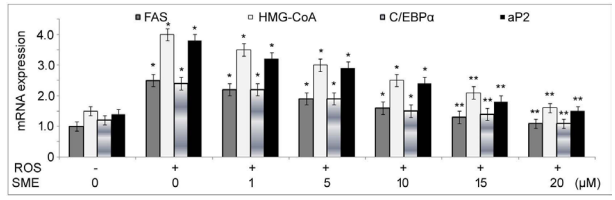

Figure 4. Effect of salicin ether on lipid metabolism related genes.
Adipocytes were treated with various concentrations of (1, 5, 10, 15, and 20 μM) salicin ether, DMSO alone (control), and/or rosiglitazone. Change in expression of FAS, C/EBPα, aP2, and HMG-CoA genes in adipocytes was determined by RT-PCR assay. *p<0.05 and **p<0.01 *vs.* control group.

Salicin ether decreases aP2, CHIP and C/EBPα protein expression

The expression of aP2, CHIP, and C/EBPα proteins was assessed in adipocytes following treatment with salicin ether by western blotting (Fig. 5). The protein expression corresponding to aP2, CHIP, and C/EBPα was markedly enhanced by rosiglitazone in adipocytes. However, treatment of adipocytes with salicin ether caused a marked reduction in rosiglitazone induced expression of aP2, CHIP, and C/EBPα proteins in concentration-based manner.

Salicin ether promotes p-Akt, p-FoxO1, p-AMPK and PPARa expression

In adipocytes, rosiglitazone administration led to a significant (p<0.05) increase in p-Akt/t-Akt expression compared to the control cells (Fig. 6). Treatment of the adipocytes with salicin ether significantly (p<0.05) reversed the rosiglitazone mediated reduction decrease in p-Akt/t-Akt expression. Moreover, rosiglitazone induced suppression of PPARa expression was also reversed in adipocytes on treatment with salicin ether. Administration of rosiglitazone to the adipocytes caused a prominent reduction in p-FoxO1/t-FoxO1 expression. However, treatment of the adipocytes with salicin ether reversed rosiglitazone mediated down-regulation of p-FoxO1/t-FoxO1 expression. Rosiglitazone administration to the adipocytes led to a significant (p<0.05) reduction in expression of p-AMPK/t-AMPK. On the other hand, salicin ether treatment significantly (p<0.05) prevented rosiglitazone mediated down-regulation of p-FoxO1/t-FoxO1 expression in adipocytes.

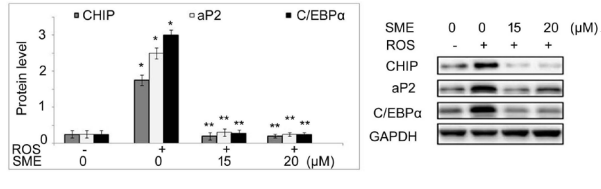

Figure 5. Effect of salicin ether on aP2, CHIP and C/EBPα proteins.
Adipocytes were treated with various concentrations of (1, 5, 10, 15, and 20 μM) salicin ether, DMSO alone (control), and/or rosiglitazone. Western blotting was performed for assessment of aP2, CHIP, and C/EBPα protein expression in adipocytes. *p<0.05 and **p<0.002 *vs.* control group.

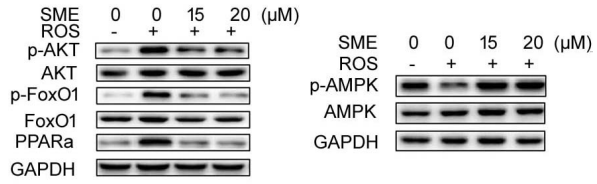

Figure 6. Effect of salicin ether on p-Akt/t-Akt and PPARa, p-FoxO1/t-FoxO1, and p-AMPK/t-AMPK in adipocytes.
Adipocytes were treated with various concentrations of (1, 5, 10, 15, and 20 μM) salicin ether, DMSO alone (control), and/or rosiglitazone. Western blotting assay was employed for assessment of protein expression in adipocytes.

DISCUSSION

Incidence of diabetes mellitus type-2 has shown an exponential increase due to changes in food habits and its morbidity rate has also increased rapidly (Lin *et al*., 2018; Wang *et al*., 2015). Patients with hyperglycemia develop several complications, such as diabetic nephropathy which advances into diabetic foot and consequently exhibits serious adverse effects on human life (Sheu *et al*., 1996). Clinicians all over the world are trying their best to understand the molecular mechanism underlying diabetes mellitus (Hongli *et al*., 2017; Jifan *et al*., 2017; Jin *et al*., 2017). Lowering of glucose absorption in human body due to insufficient insulin production leads to increased lipolysis and ultimately to hyperglycemia (Chatterjee, 2010). Hyperglycemia results in elevated glucagon production by the α-cells of pancreas, increases gluconeogenesis, and induces abnormality in glucolipid metabolism (Khan *et al*., 2003). Currently available anti-diabetic drugs are accompanied by multiple side-effects and administration of insulin causes hypoglycemia with a weight gain risk (Bøg-Hansen *et al*., 2001; Hanas & Ludvigsson, 1990). Mechanistically, oral anti-diabetics belonging to the thiazolidinedione family of compounds enhance insulin sensitivity through PPARγ, a nuclear receptor activation (Chiang *et al*., 2007). These drug molecules exhibit harmful effects including cardiovascular disorders and increase in body weight (Zhang *et al*., 2007). The modern anti-diabetic treatments have shown that down-regulation of PPARγ activity either through mutation in its active site or by genetically knocking out PPARγ decrease high fat induced insulin resistance (Evans *et al*., 2004). In the present study, salicin ether reduced PPARγ level and suppressed RXRα-PPARγ transcription in rosiglitazone exposed 293T cells. The Oil Red O staining assay showed that salicin ether suppressed formation of grease droplets in 3T3-L1 preadipocytes. Thus, salicin ether treatment caused a marked suppression in adipocyte differentiation potential. It has been shown that PPARγ plays a main role in the process of adipogenesis as well as lipogenesis (Saladin *et al*., 1999). In the present study rosiglitazone exposure led to a marked increase in PPARγ activity in 293T cells. On the other hand, rosiglitazone induced up-regulation of PPARγ activity was suppressed by salicin ether treatment in 293T cells. The elevated GLUT4 translocation to the cell membrane is accompanied by the activation of AMP protein kinase (AMPK) pathway (Welsh *et al*., 2005). GLUT4 is considered to be a therapeutic target for anti-diabetic drugs because of its involvement in glucose uptake (Welsh *et al*., 2005). It has been demonstrated that level of GLUT4 is markedly lower in animal model of diabetes (Lochhead *et al*., 2005). The present study found that salicin ether treatment promoted p-AMPK/t-AMPK level in adipocytes exposed to rosiglitazone. In rosiglitazone exposed adipocytes Akt phosphorylation was shown to be markedly lower compared to the normal adipocytes. The level of GLUT4 was also promoted in the rosiglitazone administered adipocytes on treatment with salicin ether. It is known that high blood glucose catalyses insulin release which then activates PI3K followed by Akt and FoxO1 phosphorylation (Matsuzaki *et al*., 2003). The irreversibly of phosphorylated FoxO1 promotes inhibition of glucose metabolism by insulin and production of glucose in liver (Nakae *et al*., 2001). Thus, gluconeogenesis is inhibited by insulin through Akt mediated FoxO1 phosphorylation (Oh *et al*., 2013). In the present study p-Akt/t-Akt level was markedly enhanced by salicin ether in the rosiglitazone administered adipocytes. The p-FoxO1/t-FoxO1 level in adipocytes was suppressed on administration of rosiglitazone but the effect was attenuated by salicin ether. It is evident from the current study that salicin ether exhibits anti-diabetic effect in adipocytes by activation of Akt/AMPK pathways.

CONCLUSION

In summary, the present study showed that salicin ether reduced PPARγ activity and inhibited adipocyte differentiation. Additionally, salicin ether treatment elevated activation of FoxO1/Akt/AMPK and targeted FAS/EBPα/aP2/HMG-CoA expression. Therefore, salicin ether has therapeutic importance for treatment of diabetes which needs to be investigated further.

Conflict of interest

None declared.

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