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Betel nut extract and arecoline block insulin signaling and lipid storage in 3T3-L1 adipocytes

Tusty-Jiuan Hsieh · Pei-Chen Hsieh · Ming-Tsang Wu · Wei-Chiao Chang · Pi-Jung Hsiao · Kun-Der Lin · Pong-Chun Chou · Shyi-Jang Shin

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Abstract According to several population-based studies, betel nut chewing is associated with metabolic syndrome and diabetes in British South Asians and Taiwanese. However, the underlying molecular mechanism is not yet clear. Arecoline is an alkaloid-type natural product found in betel nuts. Our aim was to clarify the influence of betel nut extract and arecoline on lipid accumulation and insulin signaling in adipocytes. We found that betel nut extract and arecoline blocked lipid storage in 3T3-L1 adipocytes. The possible mechanism may function by inhibiting the expression of the insulin receptor, glucose transporter-4, fatty acid synthase, and the lipid droplet proteins perilipin and adipophilin. In addition, betel nut extract and arecoline increased the basal level of IRS-1 serine^{307} phosphorylation and decreased insulin-stimulated IRS-1 tyrosine, Akt, and PI3 kinase phosphorylation. In conclusion, betel nut extract and arecoline have diabetogenic potential on adipocytes that may result in insulin resistance and diabetes at least in part via the obstruction of insulin signaling and the blockage of lipid storage.

Keywords Betel nut · Diabetes · Insulin resistance · IRS-1 · Perilipin

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T.-J. Hsieh · W.-C. Chang · S.-J. Shin
Department of Medical Genetics, College of Medicine,
Kaohsiung Medical University,
100 Shih-Chuan 1st Road,
Kaohsiung 80708 Taiwan, Republic of China

P.-C. Hsieh · M.-T. Wu
Graduate Institute of Occupational Safety and Health,
Kaohsiung Medical University,
100 Shih-Chuan 1st Road,
Kaohsiung 80708 Taiwan, Republic of China

M.-T. Wu
Department of Family Medicine, Kaohsiung Medical
University Hospital, Kaohsiung Medical University,
100 Shih-Chuan 1st Road,
Kaohsiung 80708 Taiwan, Republic of China

M.-T. Wu
Center of Environmental and Occupational Medicine,
Kaohsiung Municipal Hsia-Kang Hospital,
482 Shan-Ming Road,
Kaohsiung 812 Taiwan, Republic of China

P.-J. Hsiao · K.-D. Lin · P.-C. Chou · S.-J. Shin (✉)
Division of Endocrinology and Metabolism,
Kaohsiung Medical University Hospital,
Kaohsiung Medical University,
100 Shih-Chuan 1st Road,
Kaohsiung 80708 Taiwan, Republic of China
e-mail: sjshin@kmu.edu.tw

Introduction

Metabolic syndrome is a clustering of multiple metabolic abnormalities including obesity, insulin resistance, glucose intolerance, hyperinsulinemia, dyslipidemia, and hypertension (Eckel et al. 2005). A report from the DECODA Study Group indicated that the prevalence of metabolic syndrome ranges from 10% to 30% in Asian populations (The DECODA Study Group 2007).

Betel nut (*Areca catechu*) is the fourth most widely used addictive substance in the world, and most of the users are residents of Asia-Pacific regions (Boucher and Mannan 2002; Gupta and Warnakulasuriya 2002). It is well known that betel nut chewing is related to the development of oral and esophageal cancer (Wu et al. 2006; Zhang and Reichart 2007). In addition, accumulating evidence suggests that betel nut chewing contributes to the risk of metabolic syndrome and related diseases. Reports from Papua New Guinea suggest that betel nut chewing is a risk factor for diabetes and a contributing factor to poor glycemic control in diabetic patients (Benjamin 2001; Benjamin and Margis 2005). According to several population-based studies, betel nut chewing is associated with metabolic syndrome and diabetes in British South Asians and Taiwanese (Mannan et al. 2000; Chang et al. 2006; Guh et al. 2006; Yen et al. 2006). The habit of betel nut chewing independently contributes to the risk of both hyperglycemia and type 2 diabetes in Taiwanese men (Tung et al. 2004). However, the underlying molecular mechanism is not clear.

Adipocyte dysfunction has been known to link obesity to insulin resistance and type 2 diabetes (Guilherme et al. 2008). The capacity for lipid storage in adipose tissue is controlled by adipocyte hyperplasia and hypertrophy (Vázquez-Vela et al. 2008). Adiposity is increased primarily by hyperplasia during the growth stage; however, the capacity of preadipocytes to become fully functional mature adipocytes declines during aging (Vázquez-Vela et al. 2008). Even so, adipogenesis still occurs during adulthood, and the inability of an individual to undergo adipogenesis may contribute to the development of metabolic diseases (Dubois et al. 2006). White adipocytes express cell type-selective machinery that is required for triglyceride synthesis from lipoprotein-derived fatty acids as well as hormone-stimulated glucose uptake and lipolysis (Park et al. 2008). In addition, adipocytes have an endocrine function producing adipokines such as adiponectin, leptin, resistin, and visfatin, which may modulate systemic metabolism (Wajchenberg 2000; Ailhaud 2006; Waki and Tontonoz 2007; DeClercq et al. 2008). When adipose tissue reaches a threshold level of its storage capacity, the excess fat is redirected towards other organs, such as the liver, pancreas, or muscle; hence, lipotoxicity occurs in these organs and induces insulin resistance (Sethi and Vidal-Puig 2005). Furthermore, excessive accumulation of fat in adipose tissue can change the expression of inflammatory factors and adipokines, some of which modulate insulin sensitivity, not only in adipose tissue but also in other metabolically relevant organs (Sethi and Vidal-Puig 2005). Metabolic syndrome is conventionally thought to be a consequence of excessive accumulation of fat; however, evidence shows that an abnormal paucity of fat leads to elevated circulating concentrations of triglycerides and fatty acids and to insulin resistance in mice and humans (Sovik et al. 1996; Moitra et al. 1998; Shimomura et al. 1998; Laustsen et al. 2002; Huang-Doran et al. 2010). Functional adipose tissue acts as an endocrine organ and is required for the normal secretion of many metabolic-related enzymes and adipokines such as leptin and adiponectin, which enhance insulin sensitivity (Wajchenberg 2000). Lipodystrophy in humans and mice results in the impairment of leptin and adiponectin secretion and finally leads to insulin resistance and diabetes (Arioglu et al. 2000; Oral et al. 2002). Collectively, these observations demonstrate that normal insulin sensitivity and glucose homeostasis require functional adipose tissue of the proper size.

Arecoline is an alkaloid-type natural product found in betel nuts (Bhonsle et al. 1992). We hypothesized that the increased risk of insulin resistance and diabetes from betel nut chewing might result from the loss of physiological function of adipocytes, partially via arecoline. Our objectives in the present study were to clarify the influence of betel nut extract and arecoline on lipid accumulation and insulin signaling using a 3T3-L1 adipocyte model. We found that betel nut extract and arecoline have diabetogenic potential on adipocytes. Betel nut chewing may result in insulin resistance and diabetes at least in part via the obstruction of insulin signaling and the blockage of lipid storage in adipocytes.

Materials and methods

Reagents

D-Glucose, arecoline hydrobromide, 3-isobutyl-1-methylxanthine, dexamethasone, insulin, 10% formalin, Oil Red O, and propylene glycol were purchased from Sigma-Aldrich, Inc. (Saint Louis, MO, USA). Normal glucose (i.e., 5.5 mM) Dulbecco's modified Eagle's medium (DMEM; catalog no. 12320), penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Monoclonal anti-actin, perilipin A, and phosphotyrosine antibodies were purchased from Millipore (Billerica, MA, USA). Adipophilin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Preadipocyte factor 1 (Pref-1, DLK1), peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein β (C/EBPβ), fatty acid synthase (FAS), glucose transporter-4 (GLUT4), insulin receptor β subunit (IRβ), insulin receptor substrate-1 (IRS-1), phospho-IRS-1 (Ser^{307}), PI3 kinase (PI3K) p85, phospho-PI3 kinase p85 (Tyr^{458})/p55(Tyr^{199}), AKR mouse thymoma viral protooncogene (Akt), and phospho-Akt (Ser^{473}) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Protein G plus/Protein A agarose was obtained from Merck KGaA (Darmstadt, Germany). Oligonucleotides were synthesized by Bio Basic Inc. (Markham, Canada).

Preparation of betel nut extract

Betel nut extract was prepared as previously described (Jeng et al. 1994) with minor modifications. Briefly, 300 g of betel nut (*A. catechu* Linn) and 41.25 g of *Piper betle* Linn were smashed in 600 ml of double-distilled water (ddH_2O) by a blender. After crude sifting through cheesecloth to remove insoluble debris, the extract was subjected to further filtration by 6 and 3 μm filter papers to eliminate residual insoluble precipitates prior to lyophilization. The powdered extract was redissolved in ddH_2O and sterilized by 0.22 μm filters. The average quantity of arecoline in the betel nut extract was 1.53±0.75 μg/mg, which was determined by high-performance liquid chromatography.

Cell culture

3T3-L1 preadipocytes (BCRC#60159; Bioresource Collection and Research Center, Hsinchu, Taiwan) were cultured in DMEM with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After reaching 100% confluence for 2 days, 3T3-L1 preadipocytes were induced to differentiate by an induction medium containing 25 mM D-glucose, 0.32 μM insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μM dexamethasone for another 2 days. The day of induction was defined as day 0.

To investigate the influence of betel nut extract and arecoline on adipogenesis and the expression of related genes and proteins in adipocytes, we induced 3T3-L1 preadipocytes to differentiate with or without different concentrations of betel nut extract or arecoline in the induction medium for 2 days. To observe lipid droplets (LD), the cells were maintained in a medium containing 15 mM D-glucose and 0.32 μM insulin with or without different concentrations of betel nut extract or arecoline for another 4 days. Pref-1, C/EBPβ, and PPARγ are early response genes for preadipocyte differentiation, and therefore, their expressions are observed on day 2 of differentiation. IR, GLUT4, FAS, perilipin, and adipophilin are downstream targets of C/EBPβ and PPARγ (Smas et al. 1998; Ntambi and Young-Cheul 2000), so their expressions are observed when LD could be clearly seen in the cells (day 6 of differentiation).

According to the report of Hsu et al. (2010), arecoline significantly decreases insulin-induced glucose uptake only when the concentrations of arecoline are greater than 300 μM (i.e., 46.5 μg/ml). Our preliminary data showed that 300 μg/ml of betel nut extract significantly reduced the formation of LD and 20 μg /ml of arecoline presented a better effect than that of 300 μg/ml of betel nut extract. Furthermore, 300 μg/ml of betel nut extract significantly reduced the expression of C/EBPβ and PPARγ. In order to observe significant effects of betel nut extract and arecoline on insulin signaling, we, therefore, used 300 μg/ml of betel nut extract and 20 μg /ml of arecoline in this study.

Cell viability

WST-1 reagent (Roche Applied Science, Penzberg, Germany) was used to determine the toxicity of betel

nut extract and arecoline on 3T3-L1 cells. WST-1 is a tetrazolium salt that will be cleaved to formazan by mitochondrial dehydrogenases in viable cells (Ishiyama et al. 1993). To detect cell viability, 3T3-L1 cells were seeded into 96-well plates. When the density reached 80% confluence, the cells were treated with different concentrations of betel nut extract or arecoline for 24, 48, or 72 h. At the end of incubation, WST-1 was added into the cell medium for another 30 min of incubation and the amount of formazan was measured at 450 nm by an enzyme-linked immunosorbent assay (ELISA) reader.

The cytotoxicity of betel nut extract and arecoline on differentiated adipocytes was detected by using a Cytotoxicity Detection Kit^{PLUS} (Roche Applied Science, Penzberg, Germany) that is a colorimetric assay for quantification of cell death and cell lysis based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells (Racher et al. 1990). After 2 days at 100% confluence, 3T3-L1 cells were differentiated with or without betel nut extract (300 μg/ml) or arecoline (20 μg/ml). The culture media were collected after 2, 3, and 6 days of differentiation for LDH activity assay.

Oil Red O staining

Cellular LD were stained by Oil Red O (Kinkel et al. 2004). Briefly, 3T3-L1 cells were washed with PBS and fixed in 10% formalin for 1 h. After being washed with PBS, the cells were stained with Oil Red O staining solution (0.5% Oil Red O in 100% propylene glycol). After 1 h, 3T3-L1 cells were washed three times with distilled water and then observed under a phase contrast microscope. To quantify the cellular lipid amount, the stained cells were incubated with 100% isopropanol, shaken at room temperature for 20 min, and then Oil Red O in the supernatant was measured at 490 nm by an ELISA reader.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol reagent according to the supplier's protocol (Invitrogen) and quantified using the Quant-iT™ RNA Assay kit (Invitrogen). For each sample, 2 μg of total RNA were used to synthesize cDNA using the SuperScript™ III reverse transcriptase kit according to the supplier's protocol (Invitrogen). The cDNA was then diluted with water at a ratio of 1:9, and aliquots were amplified using the iQ™ SYBR Green Supermix according to the supplier's instructions (Bio-Rad Laboratories, Hercules, CA, USA). cDNA and primers were added to the PCR mixture to a final volume of 20 μl. PCR was performed using the MiniOpticon real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed using Beacon Designer 5 software (Premier Biosoft Int., Palo Alto, CA, USA) and the primer sequences are shown in Table 1. β-Actin mRNA expression was measured as an internal control.

Detection of insulin signaling and other related proteins

To test whether betel nut extract or arecoline could inhibit insulin action, we differentiated the cells with or without betel nut extract or arecoline for 2 days and incubated the cells in normal glucose DMEM without insulin for another 24 h. Then, the cells were stimulated with or without 0.32 μM insulin in DMEM containing 15 mM D-glucose for 5 or 30 min. Cells were lysed in M-PER Mammalian Protein Extraction Reagent (PIERCE) supplemented with protease inhibitors (Halt™ Protease Inhibitor Kit, PIERCE). The protein concentration was measured using the Quant-iT™ Protein Assay Kit (Invitrogen). To detect tyrosine phosphorylation on IRS-1, we immunoprecipitated IRS-1 using an anti-IRS-1 antibody and Protein G plus/Protein A agarose beads. Phosphorylated tyrosine was then detected by Western blotting. For the analysis of phosphorylated and nonphosphorylated IRS-1, Akt, and PI3K, the cell lysate was loaded and separated on sodium dodecyl sulfate–polyacrylamide gels. Protein was transferred to a polyvinylidene fluoride membrane and detected using the appropriate antibodies. The protein expressions of Pref-1, PPARγ, and C/EBPβ were detected after 2 days of differentiation; insulin receptor, GLUT4, FAS, adipophilin, and perilipin were detected after 6 days of differentiation. Images of protein expressions on the blots were quantified by ImageJ software (a public domain Java image processing and analysis program developed at the National Institutes of Health).

Statistical analysis

A minimum of three independent experiments were performed per procedure. Each sample for real-time

Table 1 Primer sequences

Gene	Sense (5′–3′)	Antisense (5′–3′)	Gene number
Pref-1	GCGTGGACCTGGAGAAAG	AGAAGTTGCCTGAGAAGCC	NM_010052
PPARγ	GGAAGCCCTTTGGTGACTTTATGG	GCAGCAGGTTGTCTTGGATGTC	NM_009883
C/EBPβ	CGCCCGCCGCCTTTAGAC	CGCTCGTGCTCGCCAATGG	NM_011146
IR	TGATGAACGGCGGACCTATGG	CTGTCCTCTGCTTGCCTCTCC	NM_010568
IRS-1	CAACAGCAGCAGCAGTCTTCC	CCGAGCCAGTCTCTTCTCTAGG	NM_010570
GLUT4	GCCAGCCTACGCCACCATAG	AGCAGAGCCACGGTCATCAAG	NM_009204
FAS	AGAGGCTTGTGCTGACTTCC	GTGGCTTCGGCGATGAGAG	NM_007988
Perilipin	GTCCCTATCCGATGCCCTGAAG	CGCTCCGCCTCTGCTGAAG	NM_175640
Adipophilin	AGTGCCCTGCCCATCATCC	GTAGTCGTCACCACATCCTTCG	NM_007408
β-Actin	GAAATCGTGCGTGACATC	CCATACCCAAGAAGGAAGG	NM_007393

PCR was analyzed in duplicate. Data were analyzed by one-way analysis of variance followed by the Bonferroni test using GraphPad Prism software. All data are presented as the mean±SD, and a probability level of $P<0.05$ was regarded as statistically significant.

Results

Effects of betel nut extract and arecoline on 3T3-L1 cell viability

After treating 3T3-L1 cells for 24 h, betel nut extract and arecoline showed no toxic effect on the cells at concentrations of up to 500 and 100 μg/ml, respectively (Fig. 1a). To further confirm the cytotoxicity, 3T3-L1 cells were treated with betel nut extract (300 μg/ml) or arecoline (20 μg/ml) for 24, 48, and 72 h. The result shows that neither betel nut extract nor arecoline significantly caused cytotoxicity for up to 72 h of incubation (Fig. 1b). Figure 1c shows that both betel nut extract and arecoline did not significantly increase LDH activity in the culture medium of adipocytes after 2, 3, and 6 days of differentiation. Our results demonstrate that the concentrations of betel nut extract and arecoline used in this paper did not cause cytotoxicity.

Betel nut extract and arecoline block the formation of lipid droplets in adipocytes

Adipocytes store dietary energy in a highly concentrated form as triglycerides, mostly in a single large LD (Rosen and Spiegelman 2006). In our experiments, we observed abundant LD in differentiated 3T3-L1 adipocytes at day 6 (Fig. 2a) compared to undifferentiated cells (Fig. 2a). Furthermore, our results showed that betel nut extract and arecoline significantly decreased LD formation in the differentiated adipocytes (Fig. 2a). The cellular lipid amount was quantified and converted to a percentage. Betel nut extract, concentrations from 100 to 300 μg/ml, significantly decreases the cellular lipid amount in a dose-dependent manner in adipocytes (Fig. 2b). Arecoline (1, 10, and 20 μg/ml) also decreased lipid amount in a dose-dependent manner (Fig. 2b).

Effects of betel nut extract and arecoline on the differentiation of adipocytes

To explore whether betel nut extract and arecoline could impair adipocyte differentiation, we detected the mRNA and protein expressions of the preadipocyte marker Pref-1 (Smas et al. 1998). Figure 3a, b shows that the Pref-1 mRNA and protein were dramatically decreased in the differentiated adipocytes treated with or without the betel nut extract and arecoline after 2 days of differentiation. However, arecoline could partially reverse the downregulation of Pref-1 mRNA and protein (Fig. 3a–c). Even though betel nut extract did not significantly reverse the downregulation of Pref-1 mRNA, we still observed a trend that the expression of Pref-1 mRNA was higher in the group with betel nut extract treatment than the group without the treatment (Fig. 3a). In contrast, betel nut extract significantly reversed the downregulation of Pref-1 protein (Fig. 3b, c). Our results

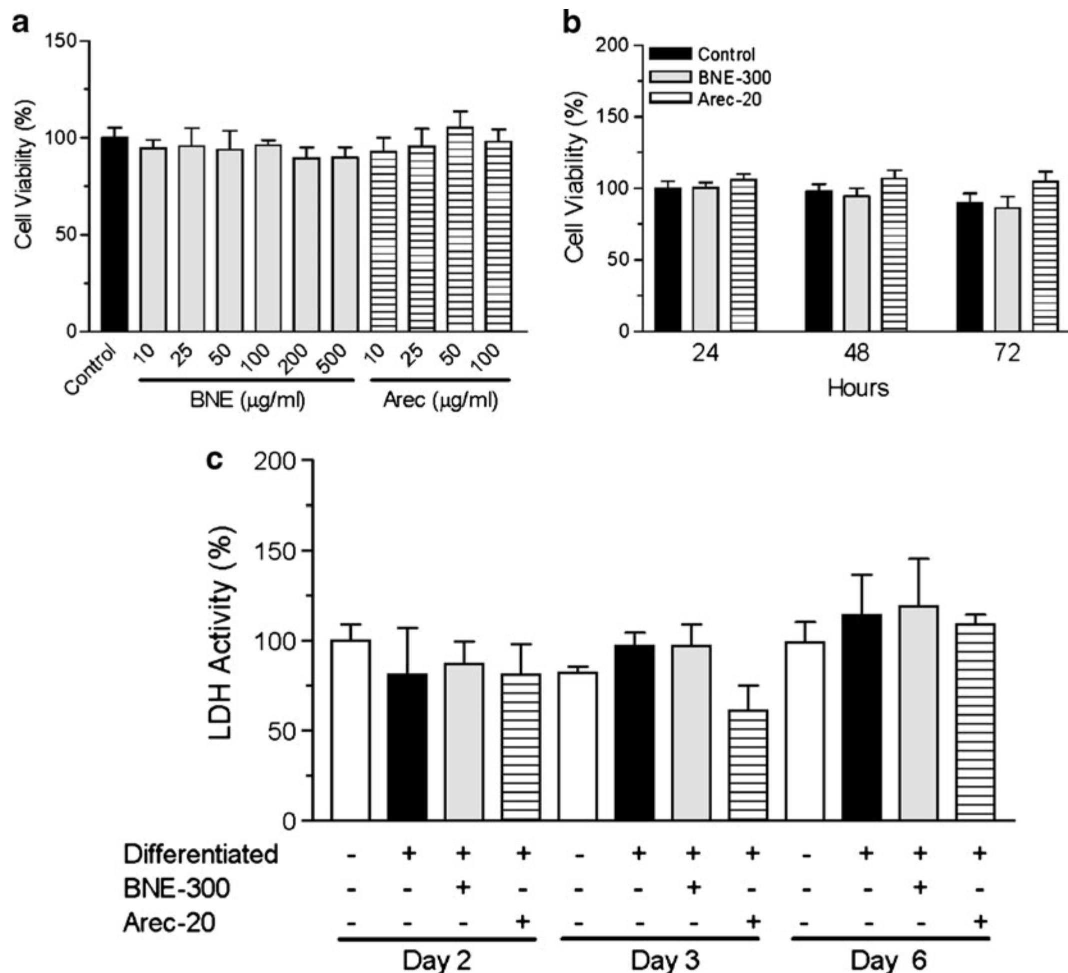

Fig. 1 Effects of betel nut extract and arecoline on cell viability of 3T3-L1 cells. **a** Undifferentiated 3T3-L1 cells were treated with different concentrations of betel nut extract (*BNE*) and arecoline (*Arec*) for 24 h. **b** Undifferentiated 3T3-L1 cells were treated with betel nut extract (300 μg/ml) and arecoline (20 μg/ml) for 24, 48, and 72 h. **c** LDH activity in the culture medium. 3T3-L1 cells were treated with betel nut extract (300 μg/ml) and arecoline (20 μg/ml) for 2, 3, and 6 days. Data are presented as the mean±SD from six independent experiments

indicate that betel nut extract and arecoline may interfere with the differentiation of adipocytes.

Effects of betel nut extract and arecoline on the expression of C/EBPβ and PPARγ

C/EBPβ and PPARγ are transcriptional factors that regulate the expression of genes related to adipogenesis (Ntambi and Young-Cheul 2000). Our results show that the mRNA and protein expressions of C/EBPβ and PPARγ were significantly increased in the differentiated adipocytes after 2 days of differentiation (Fig. 4a–e). In contrast, the treatment of betel nut extract and arecoline significantly inhibited the mRNA and protein expressions of C/EBPβ and PPARγ (Fig. 4a–e).

Effects of betel nut extract and arecoline on lipid storage-related genes

Insulin promotes glucose transport into adipocytes via GLUT4 and increases triglyceride accumulation via PPARγ-activated FAS (Ntambi and Young-Cheul 2000). Perilipin and adipophilin are essential for controlling lipid storage and lipolysis (Heid et al. 1998; Wolins et al. 2005; Miyoshi et al. 2010). Hence, we detected the expression of these genes in order to clarify the molecular mechanism of betel nut extract

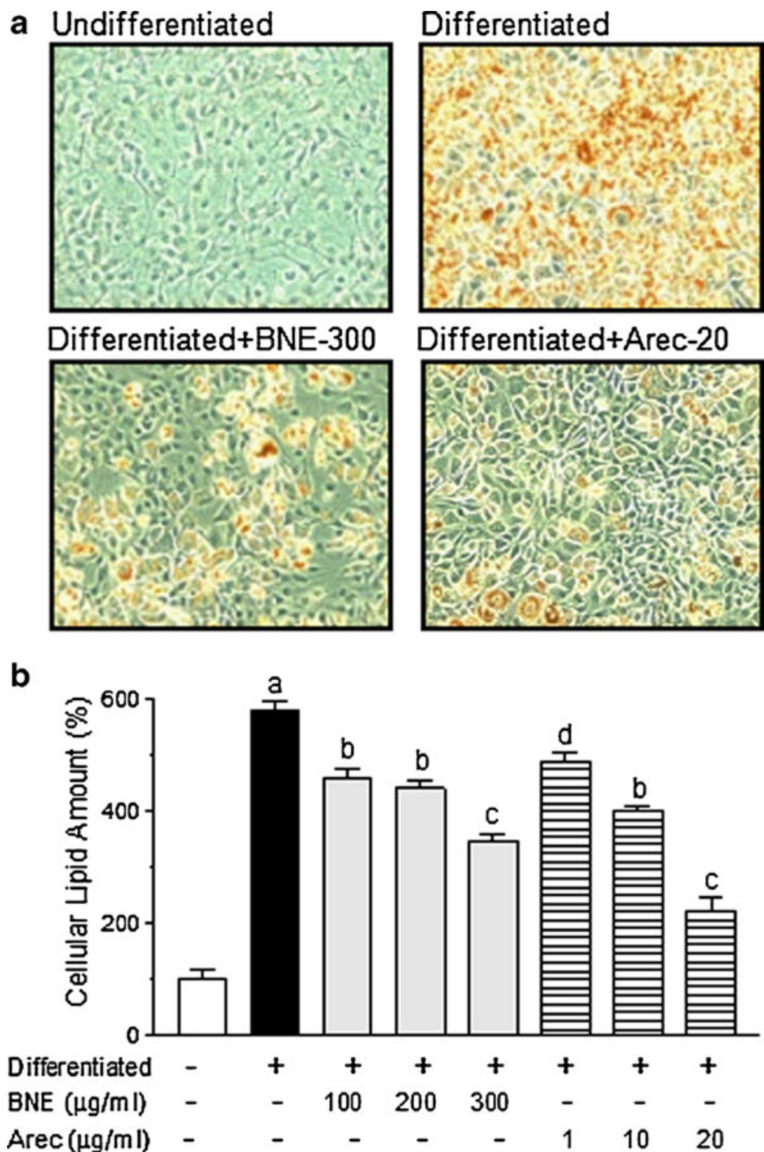

Fig. 2 Effects of betel nut extract and arecoline on lipid accumulation in adipocytes. **a** Cellular LD were stained by Oil Red O (*red color*) after 6 days of differentiation with or without the treatment of 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline (*Arec*). **b** The cellular lipid was quantified after 6 days of differentiation with or without different concentrations of betel nut extract (100, 200, and 300 μg/ml) or arecoline (1, 10, and 20 μg/ml) by dissolving Oil Red O in isopropanol. Data are presented as the mean± SD from four independent experiments. *a* $p<0.001$ compared to undifferentiated group, *b* $p<0.01$ compared to differentiated adipocytes, *c* $p<0.001$ compared to differentiated adipocytes, *d* $p<0.05$ compared to differentiated adipocytes

and arecoline on the inhibition of adipogenesis. Table 2 shows that the mRNA expressions of the insulin receptor, IRS-1, GLUT4, FAS, perilipin, and adipophilin were significantly downregulated by treatment with betel nut extract (300 μg/ml) and arecoline (20 μg/ml) when compared to differentiated adipocytes. Figure 5 demonstrates that the protein expressions of the insulin receptor, GLUT4, FAS, perilipin, and adipophilin were decreased by treatment with betel nut extract and arecoline in adipocytes, which indicates that betel nut extract and arecoline could affect these genes and then interfere in adipogenesis.

Effects of betel nut extract and arecoline on insulin signaling

IRS-1, PI3K, and Akt are the downstream effectors of insulin receptor. The activation of insulin receptor leads to the tyrosine phosphorylation of IRS-1, which subsequently activates PI3K and Akt to regulate glucose and lipid metabolism (Hill et al. 1999; Foran et al. 1999; Saltiel and Kahn 2001). In contrast, evidence has shown that phosphorylation of serine^{307} on IRS-1 blocks the interaction with the insulin receptor and inhibits insulin action (Aguirre et al. 2002). Our results show that the basal level of

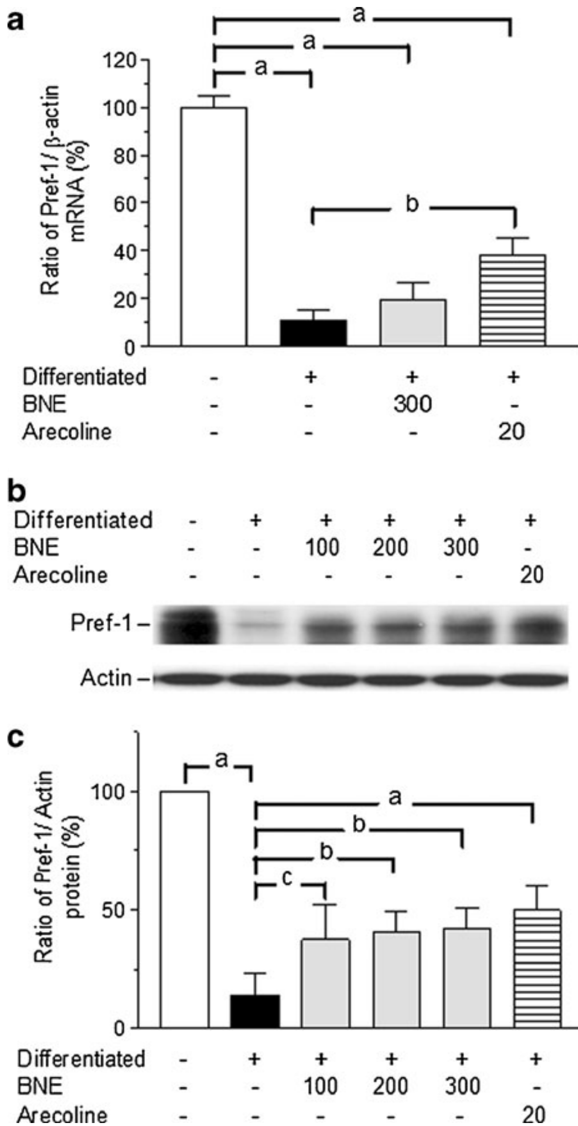

Fig. 3 Effects of betel nut extract and arecoline on Pref-1 expression in adipocytes. **a** Pref-1 mRNA was detected after 2 days of differentiation with or without 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline by real-time PCR. Each sample was performed in duplicate for real-time PCR. **b** Pref-1 protein was detected after 2 days of differentiation with or without 100, 200, and 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline by Western blotting. **c** Densitometry data of the blots. Data are presented as the mean±SD from three independent experiments. *a* $p<0.001$, *b* $p<0.01$, *c* $p<0.05$

serine^{307}-phosphorylated IRS-1 in differentiated adipocytes was lower than that of undifferentiated preadipocytes (Fig. 6a, b). In contrast, the differentiated adipocytes treated with the betel nut extract and arecoline expressed a higher basal level of serine^{307}-phosphorylated IRS-1 than that of untreated adipocytes (Fig. 6a, b). This result implies that betel nut extract and arecoline may inhibit insulin action in adipocytes. Figure 6c, d shows that insulin increased tyrosine-phosphorylated IRS-1 in adipocytes. Treatment with betel nut extract and arecoline blocked insulin-stimulated tyrosine phosphorylation on IRS-1 (Fig. 6c, d). In contrast, insulin did not affect the phosphorylation of serine^{307} on IRS-1 (Fig. 6c, e). We observed a decreased trend of the total IRS-1 in the betel nut extract- and arecoline-treated groups; however, the difference between groups did not reach a significant level (Fig. 6f). The levels of phosphorylated PI3K p85 protein and Akt were increased by insulin stimulation in adipocytes, whereas the phosphorylation was inhibited by treatment with betel nut extract and arecoline (Fig. 7a–c). Our results indicate that betel nut extract and arecoline impair insulin signaling in adipocytes.

Discussion

It has been estimated that more than 10% of the world's population are regular consumers of betel nuts (Croucher and Islam 2002), which can have profound impacts on public health issues, including metabolic syndrome. Although cumulative population-based studies suggest that betel nut chewing is a risk factor for metabolic syndrome and type 2 diabetes (Benjamin 2001; Mannan et al. 2000; Tung et al. 2004; Chang et al. 2006; Guh et al. 2006; Yen et al. 2006), to date, the direct link and its related molecular pathways remain a significant literature gap. In this study, we provide evidence that betel nut extract and arecoline could impair insulin signaling, which may weaken the function of the adipocyte as a depot of lipid. Betel nut extract and arecoline have several diabetogenic effects on adipocytes, including (1) an increase in IRS-1 serine^{307} phosphorylation, (2) blocking of the insulin-stimulated IRS-1 tyrosine, PI3K, and Akt phosphorylation, and (3) a decrease in the expression of insulin receptor, GLUT4, FAS, and LD proteins. Our results present the underlying molecular mechanisms that may partially explain the link between betel nut chewing and metabolic syndrome.

Nitrosamines in smoked cured mutton were reported to have a possible diabetogenic effect

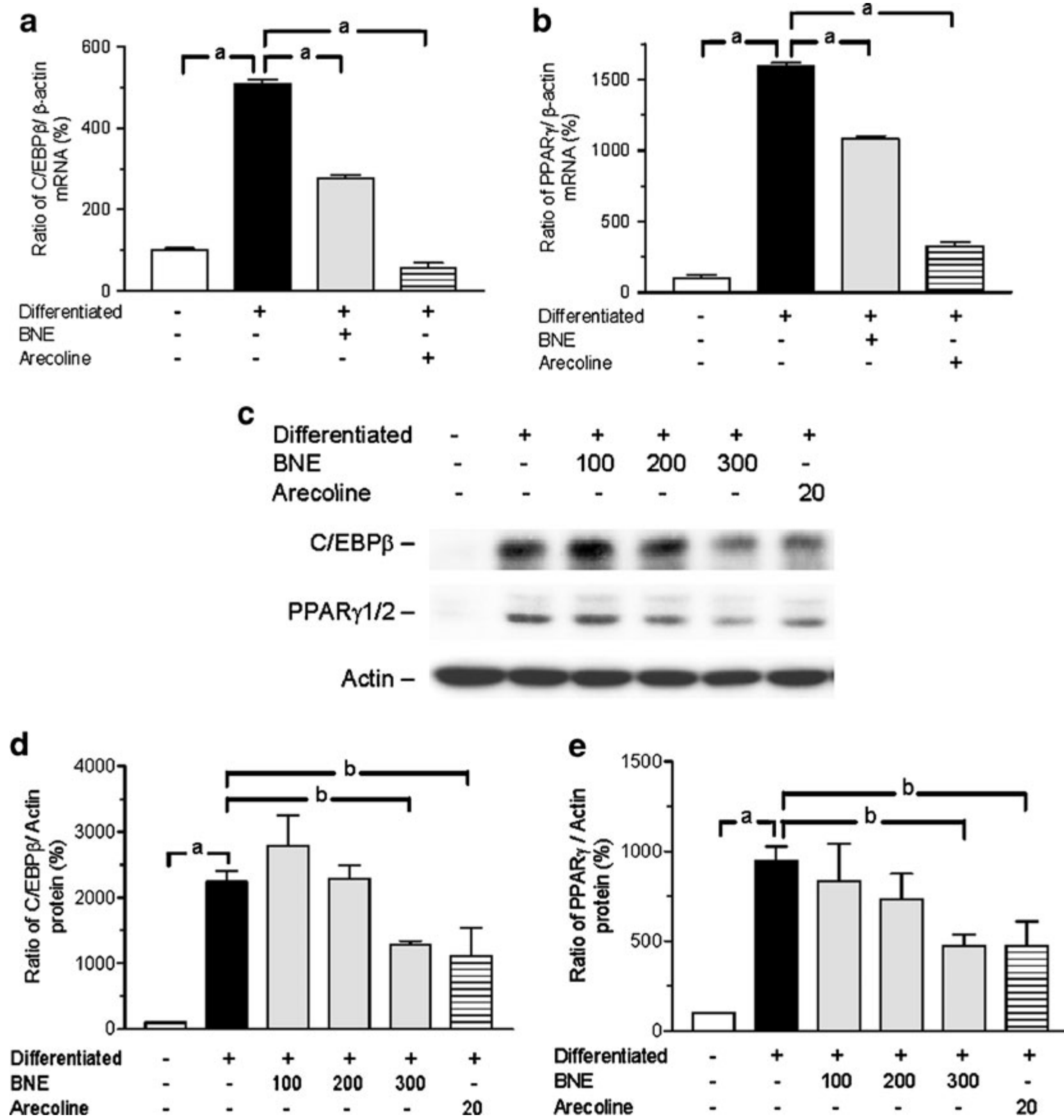

Fig. 4 Effects of betel nut extract and arecoline on C/EBPβ and PPARγ expressions in adipocytes. **a**, **b** C/EBPβ and PPARγ mRNAs were detected after 2 days of differentiation with or without 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline by real-time PCR. Each sample was performed in duplicate for real-time PCR. **c** C/EBPβ and PPARγ proteins were detected after 2 days of differentiation with or without 100, 200, and 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline by Western blotting. **d**, **e** Densitometry data of the blots. Data are presented as the mean±SD from three independent experiments. *a* $p<0.001$, *b* $p<0.01$

(Stowers and Ewen 1991). This report let Boucher et al. (1994) consider the betel nut as a diabetogenic substance because the betel nut is also a potent source and precursor of nitrosamines. According to the study of Boucher et al., betel nut consumption results in glucose intolerance in adult CD1 mice and in their F1 and F2 offspring. Furthermore, the mean islet areas are increased in offspring of betel-fed parents (Boucher et al. 1994). This is the first report that uses animal models and directly proves the diabetogenic effect of betel nut extract. Boucher et al. also suggest that not all of the directly diabetogenic effect of betel nut powder is due to readily extractable nitrosamines. Thus far, what compounds in betel nuts may have diabetogenic effects is still not fully understood. Arecoline is another natural product found in betel nuts (Bhonsle et al. 1992) and it has been reported to inhibit adipogenesis of 3T3-L1 cells

Table 2 Effects of betel nut extract (BNE) and arecoline on the mRNA expression of 3T3-L1 adipocytes

Gene	Adipocyte	Adipocyte+BNE (300 μg/ml)	Adipocyte+ arecoline (20 μg/ml)
IR	100	58±17.5*	73±12.9**
IRS-1	100	70±12.0**	30±19.9***
GLUT4	100	36±10.4***	21±8.8***
FAS	100	46±4.0***	20±10.6***
Perilipin	100	91±2.8**	46±4.1***
Adipophilin	100	64±7.2***	39±9.5***

The mRNA expression of the adipocyte group was assigned 100%, and all other values were presented as relative percentages. All data are presented as the mean±SD; $n=4$ for each group

*$p<0.01$, **$p<0.05$, ***$p<0.001$ compared to the adipocyte group

(Hsu et al. 2010). In our study, we demonstrate that betel nut extract and arecoline reduced LD accumulation in adipocytes. Our observation is consistent with the report from that of Hsu et al. According to our measurement, the quantity of arecoline in 300 μg of betel nut extract is around 0.234–0.684 μg. Compared to 1 μg/ml of arecoline, 300 μg/ml of betel nut extract has a better effect to reduce cellular lipid amount, indicating that other compounds in betel nut extract may contribute to the inhibition of lipid accumulation in adipocytes. The reduction of cellular LD does not result from the toxicity of betel nut extract and arecoline because we did not observe changes in cell viability when cells were stimulated with high concentrations of betel nut extract (500 μg/ml) and arecoline (100 μg/ml). We also did not detect an increase of LDH activity released from adipocytes that were incubated with betel nut extract (300 μg/ml) and arecoline (20 μg/ml) for 6 days.

Pref-1 is a preadipocyte marker and an inhibitor of adipocyte differentiation (Smas et al. 1998; Sul et al. 2000). In this study, we demonstrate that the mRNA and protein expressions of Pref-1 were dramatically decreased in the adipocytes after 2 days of differentiation. This result indicates that the preadipocytes are totally transformed to adipocytes in 2 days. Treatment with betel nut extract and arecoline partially reversed the decrease in Pref-1 expression, suggesting that these two substances may interfere in the differentiation of preadipocytes. C/EBPβ and PPARγ are two important transcription factors that regulate the expression of genes related to glucose uptake, triglyceride synthesis, and LD formation in adipocytes (Ntambi and Young-Cheul 2000). These two transcription factors will be activated at a very early stage of differentiation and regulate downstream genes. Our results show that betel nut extract and arecoline treatment could decrease the expression of C/EBPβ and PPARγ, which may be another critical factor that inhibits lipid storage in adipocytes.

The adipocyte is a key component in the control of energy balance and in lipid homeostasis (Guilherme et al. 2008). In particular, the white adipocyte expresses cell type-selective machinery required for triglyceride synthesis from lipoprotein-derived fatty acids as well as hormone-stimulated glucose uptake and lipolysis (Park et al. 2008). LD are energy-storage organelles with a surprisingly complex function in lipid homeostasis. Cells store nonesterified fatty acids as triacylglycerol in LD (Murphy and Vance 1999). The LD is surrounded by a phospholipid monolayer to which a number of proteins bind and thereby participate in the regulation of lipid fuel metabolism (Londos et al. 1999). The protein coat of LD contains at least one member of the PAT family consisting of perilipin, adipophilin, TIP47, and S3-12 protein (Miura et al. 2002). Recently, it has been suggested that LD proteins potentially play a key role in the development of insulin resistance and human diabetes (Guilherme et al. 2008). The major function of perilipin is to regulate lipolysis by protecting the intracellular LD from neutral lipases within the cell under basal conditions (Brasaemle et al. 2000; Souza et al. 2002; Tansey et al. 2003). It has been found that perilipin null mice exhibit diminished adipose tissue and increased insulin resistance (Saha et al. 2004). Like perilipin, adipophilin shields triglyceride stores from cytosolic lipases, although less effectively (Larigauderie et al. 2006). Our result demonstrates that betel nut extract and arecoline significantly decreased the mRNA and protein expressions of the insulin receptor, GLUT4, FAS, perilipin, and adipophilin in differentiated adipocytes. This result indicates that betel nut extract and arecoline influence adipogenesis via the regulation of these genes. Therefore, the reduction in LD in adipocytes by betel nut extract and arecoline may be regulated by at least two mechanisms. First, the use of

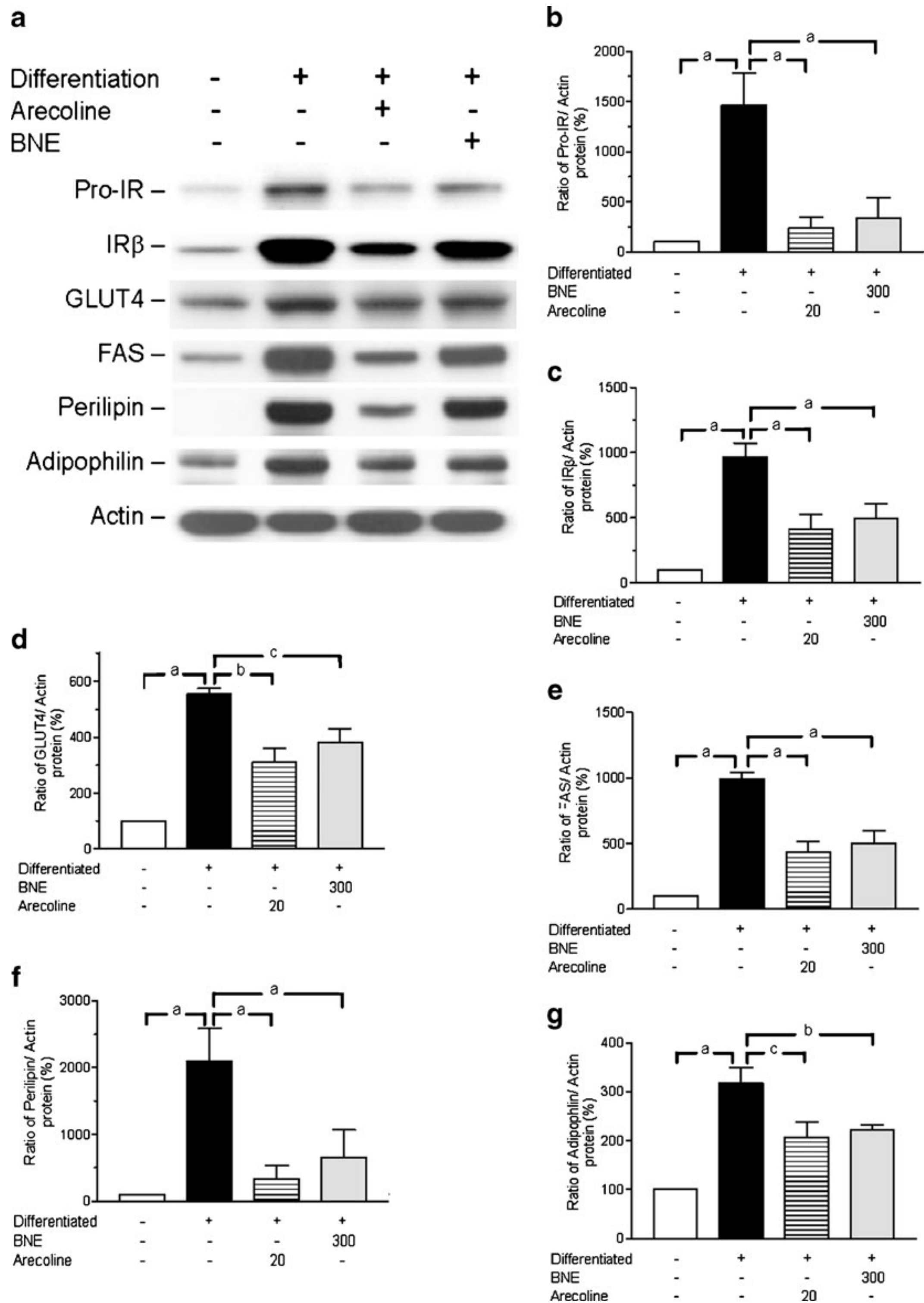

Fig. 5 Effects of betel nut extract and arecoline on the protein expression of insulin receptor, GLUT4, FAS, perilipin, and adipophilin in adipocytes. **a** Insulin receptor (pro-IR and IRβ subunit), GLUT4, FAS, perilipin, and adipophilin proteins were detected after 6 days of differentiation with or without 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline by Western blotting. **b**–**g** Densitometry data of the blots. Data are presented as the mean±SD from three independent experiments. *a* $p<0.001$, *b* $p<0.01$, *c* $p<0.05$

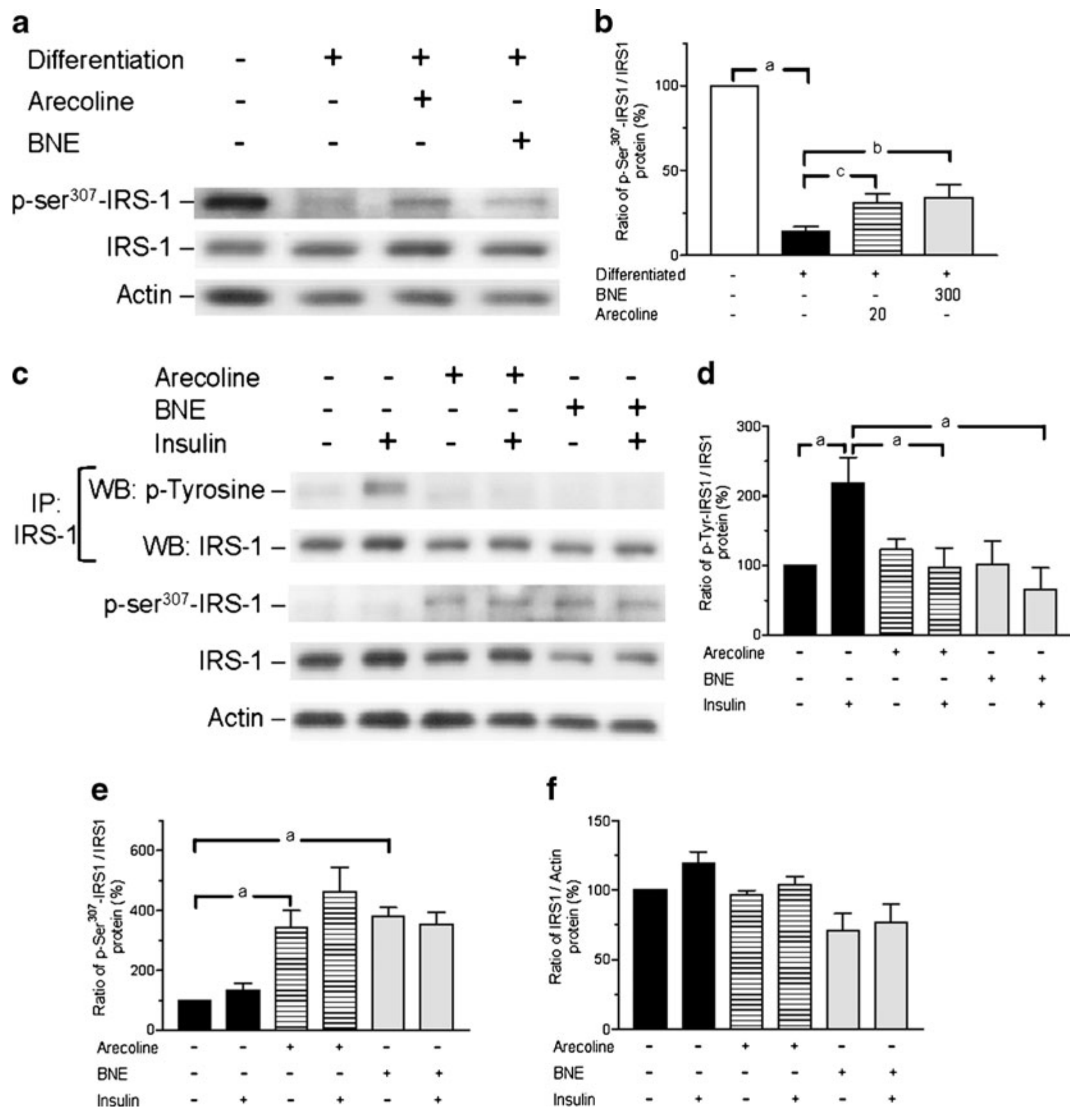

Fig. 6 Effects of betel nut extract and arecoline on the insulin signaling pathway in adipocytes. **a** Serine[307]-phosphorylated IRS-1 and IRS-1 proteins were detected after 6 days of differentiation with or without 300 μg/ml betel nut extract (*BNE*) or 20 μg/ml arecoline by Western blotting. **c** Tyrosine-phosphorylated IRS-1, serine[307]-phosphorylated IRS-1, and IRS-1 proteins were detected after 5 min of insulin stimulation. **b**, **d**–**f** Densitometry data of the blots. Data are presented as the mean±SD from three independent experiments. *a* $p<0.001$, *b* $p<0.01$, *c* $p<0.05$

glucose as a source of nonesterified fatty acids and triacylglycerol to form LD may be decreased via downregulation of insulin receptor, GLUT4, and FAS. Hsu et al. (2010) has reported that arecoline could decrease glucose uptake in 3T3-L1 adipocytes. Second, lipolysis may be promoted by removing the protection from cytosolic lipases via diminishing perilipin and adipophilin on LD. Hsu et al. (2010) also reported that arecoline increased lipolysis in 3T3-L1 adipocytes.

Insulin resistance results from a complex interplay among nutrient overload, systemic fatty acids excess, inflammation of the adipose tissue, the endoplasmic reticulum, and oxidative stresses (Hotamisligil 2006). Abnormal regulation of insulin signaling is an important factor in the development of insulin resistance. Insulin initiates its metabolic effects, including glucose transport, by binding to the insulin receptor. This event initiates a cascade of cell-

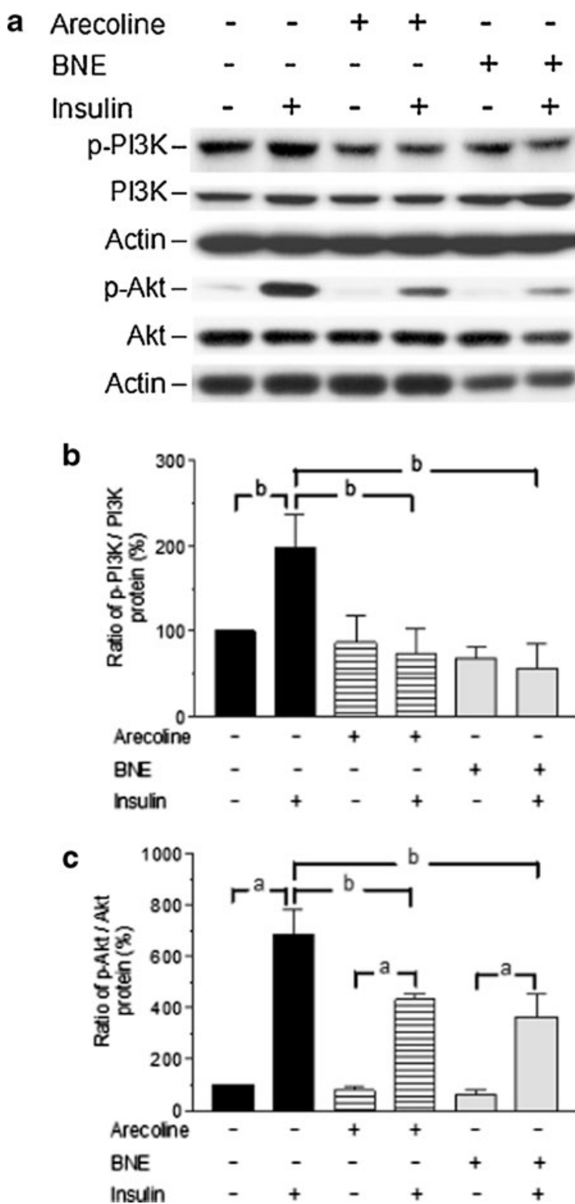

Fig. 7 Effects of betel nut extract and arecoline on the insulin signaling pathway in adipocytes. **a** Phosphorylated and non-phosphorylated PI3K p85 proteins and Akt were detected after 30 min of insulin stimulation. The data represent three independent experiments. **b**, **c** Densitometry data of the blots. Data are presented as the mean±SD from three independent experiments. *a* $p<0.001$, *b* $p<0.01$

signaling responses, including phosphorylation of the insulin receptor substrate (IRS) 1–4 proteins that act as docking proteins for a number of downstream effector molecules, such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Saltiel and Kahn 2001). Tyrosine phosphorylation of IRS-1 permits its association with the p85 subunit, which in turn activates PI3K and its effector Akt/protein kinase B, a putative mediator of the effects of insulin on glucose transport (Hill et al. 1999; Foran et al. 1999). In addition to tyrosine phosphorylation, IRS-1 is susceptible to serine phosphorylation; in particular, it has been suggested that the phosphorylation of serine^{307} attenuates insulin signaling (Paz et al. 1997; Rui et al. 2001). One of our concerns about the effect of betel nut extract and arecoline on the action of insulin in adipocytes is whether it could change the downstream signaling of the insulin receptor. Our results demonstrate that betel nut extract and arecoline treatment maintain a higher basal level of serine^{307}-phosphorylated IRS-1 protein in differentiated adipocytes. Furthermore, the treatment blocked insulin-stimulated IRS-1 tyrosine, PI3K p85 protein, and Akt phosphorylation. These findings offer evidence that betel nut extract and arecoline could decrease insulin sensitivity in adipocytes.

Obesity is one of the major contributors for the metabolic syndrome (Dubois et al. 2006) and betel nut chewing is reported to be associated with general and central obesity (Lin et al. 2009). These reports are contradictory to our observation that arecoline and betel nut extract inhibit the formation of LD in adipocytes. However, metabolic syndrome is a complex disease which involves the interaction between environments and genetic factors. The mechanism that links betel nut and metabolic syndrome could not be fully explained via any signal experimental model. Our results could only explain the effect and the potential mechanism of betel nut extract on adipogenesis in adipocytes. To further clarify the effect of betel nut on obesity and metabolic syndrome, appropriate animal studies should be established. Furthermore, other compounds in betel nuts should also be tested for their diabetogenic effects.

In conclusion, betel nut extract and arecoline not only affect the expression of critical genes related to adipogenesis but also directly impair insulin signaling in adipocytes. Our findings may explain, at least in part, the link between betel nut chewing and metabolic syndrome.

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