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Epigallocatechin Gallate (EGCG) and Rutin Suppress the Glucotoxicity through Activating IRS2 and AMPK Signaling in Rat Pancreatic β Cells

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Pancreatic β cell failure is one critical metabolic disorder in the development of type 2 diabetes. Decreased viability and dysfunction of β cells would accelerate the diabetic pathogenesis associated with higher mortality. In this study, the tea polyphenol EGCG (epigallocatechin gallate) and the buckwheat flavonoid Rutin were investigated to attenuate the induced glucotoxicity in β cells. EGCG and Rutin could preserve the insulin secretory machinery and stimulate insulin receptor substrate 2 (IRS2) signaling in rat pancreatic β cells, RIN-m5F. These findings further demonstated the reduced glucolipotoxic effects of EGCG and Rutin through activating AMP-activated protein kinase (AMPK) signaling to inhibit the activities of lipogenic enzymes and ameliorating mitochondrial function. Consequently, the cell viability was retained after attenuating the glucotoxicity through the broad effect of EGCG and Rutin. The intrinsic protective effects of EGCG and Rutin in preserving the insulin signaling and regulating lipogenesis, manipulating cell cycling, and maintaining mitochondrial function to achieve the integrity of β cells, which highlight the possibilities of EGCG and Rutin as novel strategies for the prevention of type 2 diabetes.

KEYWORDS: AMPK; β cell; EGCG; glucotoxicity; IRS2; Rutin

INTRODUCTION

Diabetes mellitus is the most common metabolic disorder that causes about 5% of all death globally each year. The etiology of diabetes could be genetics or environmental influences (*1*), in which type 2 diabetes comprises 90% of diabetic individuals. Two major characteristics of type 2 diabetes are pancreatic β cell dysfunction and peripheral insulin resistance (*2*), which both could arise from an imbalanced energy metabolism, while which one is the cause or the consequence of type 2 diabetes still needs to be resolved. Indeed, pancreatic β cells play a critical role in the pathogenesis of type 2 diabetes even while the controversy still stands. The failure of pancreatic β cell after adaptation resulted from high metabolic demand, leading to a decompensation and acceleration of the progression of diabetes, accompanied with a higher mortality (*3*). All the therapies of type 2 diabetes are still hampered by the complication of the metabolic disorder, therefore, further investigation on the molecular mechanism of this disease is needed. The pancreatic β cell endocrine system might be a good starting point for exploring the molecular alteration in the development of diabetes.

Tea (*Camellia sinensis*) was regarded as a potential agent with antidiabetes and antiobesity activity (*4*). Previous studies have revealed EGCG, the most abundant polyphenol in green tea, attenuated the IRS1 signaling and lipid accumulation in hepatocytes (*5*, *6*). IRS proteins are major mediators of insulin action, regulating cell survival and cell proliferation; the conduction of IRS2 was particularly implicated in the whole endocrine system (*7*). Disruption of mice IRS2, not IRS1, would lead to the spontaneous apoptosis of β cells and to evolve into individuals with the similarities of diabetes (*8*); IRS2 acts critically in β cells, which could activate downstream signaling, Akt, FoxO1 (forkhead-O transcription factor 1), and PDX-1 (pancreas-duodenum homeobox-1), and preserve the integrity of β cells during the high metabolic demand. The chronic metabolic stress from overnutrition compromised IRS2 signaling and diminished Akt activity (*9*), indicating a decrease in the insulin secretion and to activate FoxO1 (*10*); consequently, PDX-1, the major insulin transcription factor, would fail to respond to the metabolic demand (*11*). EGCG has been examined as Akt activator in hepatocytes under the insulin resistance condition (*6*) and the ability of insulin mimicry (*12*), addressing the potential of the polyphenol in manipulating the insulin signaling and the protection of an overloaded metabolism.

Obesity is one well-known risk factor for the development of metabolic syndrome, causing the disturbance of the metabolism. Hyperglycemia is regarded as an undoubtable contributor for diabetes, and furthermore, that results in upregulating the cellular lipogenesis (*13*), deteriorating the peripheral insulin sensitivity and pancreatic insulin secretory capacity (*14*). Theaflavins (the major black tea polyphenol) and EGCG with the attenuation of lipogenesis and insulin signaling, respectively, in hepatocytes depend on AMPK activation. Suppression of AMPK from

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glycolipotoxicity or lipotoxicity might cause the multiple metabolic abnormalities would be critically concerned to balance the metabolic harmony (*15*). Previously, EGCG has been investigated in a type 1 diabetic animal model, a streptozotocin-induced diabetic rat, however, the data indicated EGCG appeared to express controversial effects on preventing the onset of autoimmune diabetes (*16*, *17*). Nevertheless, other in vivo and in vitro experiments demonstrated that as nutritional supplementation, EGCG could maintain the insulin secretory function and expressed antiapoptosis activity in isolated islets, and moreover, EGCG could improve the glucose and lipid metabolism in preclinical type 2 diabetic models, *db*/*db* mice and Zucker Diabetic Fatty (ZDF) rat (*18*, *19*), implicating the protective role of EGCG in pancreatic islets and attenuating the pathological severity of type 2 diabetes. Rutin, a flavonoid from buckwheat (*Fagopyrum esculentum*), has been shown the ability to reduce blood glucose concentration in diabetic model rats (*20*), yet the underlying molecular mechanism has not been elucidated. In this current study, two natural occurring compounds, EGCG and Rutin, and their antiglucotoxic effects in rat pancreatic β cells have been investigated and the molecular mechanism of protection has been validated. Our results implicated that EGCG and Rutin could increase the viability, preserve the insulin secretory machinery, and reduce intracellular lipid accumulation under high glucose condition, achieving a comprehensive protection of pancreatic β cell to manifest the antidiabetic benefits of natural tea and buckwheat.

MATERIALS AND METHODS

Materials. The pure compound (−)-epigallocatechin-3-gallate (EGCG), Rutin, D-glucose, sodium pyruvate, HEPES, dichlorofluorecin diacetate (DCFH-DA), 3,3′-dihexyloxacarbocyanine iodide (DiO6), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). The Mitotracker dye was purchased from Invitrogen Corporation (Carlsbad, CA). The anti-PDX1, anti-SREBP1, anti-pTyr, and anti-LKB1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-pAkt (Ser473) and anti-Bax were obtained from Cell signaling Technology, Inc. (Beverly, MA). The anti-pAMPK (Thr172), anti-AMPK, anti-IRS2, and anti-p21 were from Upstate Biotechnology (Lake Placid, NY). The anti-FAS and anti-Cyclin D1 were from BD Bioscience (Franklin Lakes, NJ). The anti-pFoxO1 (Ser256), anti-FoxO1, and anti-pSer were obtained from Abcam Inc. (Cambridge, MA). The anti-pACC (Ser79) was purchased from Transduction Laboratory (Lexington, KY).

Cell Culture. RIN-m5F rat insulinoma pancreatic β cells were obtained from the NHRI cell bank (National Health Research Institutes, Taiwan) and maintained in RPMI 1640 containing 11 mM glucose supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, UT) in a humidified 5% CO_2 incubator at 37 °C.

Immunoprecipitation and Western Blotting. Cells were incubated in media containing 11 mM glucose followed by a 2 h starvation and treated with 33 mM glucose contained media (defined as high glucose stimulation) with or without EGCG or Rutin for an indicated duration of time. Cells were lysed with buffer (10% glycerol, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5 mM phenylmethysulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.5 mM dithiothreitol), the lysates were centrifuged at 12000 rpm for 30 min, and then the supernatants were collected as whole cell extracts. For Western blotting, equal amounts of total cellular protein (20 μg) were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Millipore), and probed with a primary antibody, followed by a secondary antibody conjugated with horseradish peroxidase. The immunocomplexes were visualized with an enhanced chemiluminescence system (Perkin-Elmer life sciences, Boston, MA). For immunoprecipitation, equal amounts of cell lysates were precipitated with anti-IRS2 antibodies and then immobilized on protein G-Sepharose beads followed by gentle rocking overnight at 4 °C. The immunocomplexes were collected by centrifugation at 12000 rpm for 30 min at 4 °C, washed with ice-cold PBS, and incubated at 100 °C for 10 min with 20 μL of electrophoresis buffer, and the supernatant was analyzed by Western blotting.

ATP Detection Assay and Insulin Secretion Detection. The cells were preincubated in the media containing low glucose condition (3.3 mM), maintained glucose condition (11 mM), or high glucose condition (33 mM) for 48 h, and then tthe cells were stimulated by replacing the media with media containing high glucose condition with or without Rutin or EGCG. After a 2 h stimulation, the cell media were quantified by rat insulin ELISA kit (Mercodia Inc., Uppsala, Sweden), and the ATP contents were quantified by luminescence ATP detection assay system (Perkin-Elmer life sciences, Boston, MA).

Immunocytochemistry. Cells were incubated in high glucose condition with or without Rutin or EGCG for 72 h. Cells were washed with PBS, fixed with 10% formalin for 30 min on ice, washed twice with PBS, blocked with 1% BSA/PBS for 1 h at room temperature, and then incubated with anti-PDX1 antibodies (100X) at room temperature for 1 h. The secondary antibody conjugated with FITC (200X, Santa Cruz biotechnology) was used for a 1 h incubation at room temperature. The nuclei were stained by DAPI for 1 h and all images were acquired from Leica TCS SP5 confocal microscopy (Leica Microsystems GmbH, Wetzlar, Germany).

Assay for ROS, Mitochondrial Membrane Potential (MMP) and Mitotracker Staining. Cells were incubated in media of 33 mM glucose with or without EGCG or Rutin for the indicated duration and then stained with DCFH-DA (ROS indicator) or DiO6 (MMP -sensitive dye) or Mitotracker for another 30 min. Cells were washed with ice-cold PBS to remove excessive fluorescence dye and resuspended in PBS for analysis. The fluorescence retention of survival cells was detected through a BD FACSCalibur system (BD Biosciences, Mountain View, CA).

Oil Red O Staining. To measure the cellular lipid droplet accumulation, cells were incubated in a maintained glucose condition or high glucose condition with or without Rutin or EGCG after a 2 h starvation. These cells were incubated for 5 days and the media was replaced every 24 h. The staining procedure followed was from a previous study (*5*).

MTT Assay. Cells were incubated in media of 33 mM glucose with or without Rutin or EGCG after a 2 h starvation. The cell mass was examined by the MTT assay at the indicated duration of time. The MTT working solution (2 mg/mL in PBS) was exposed to each cultured well and incubated for 2−4 h at 37 °C. The MTT-formazan crystals were dissolved in 1 mL of DMSO and the absorbance of 550 nm was performed by spectrophotometer.

Statistical Analysis. All results were expressed as means ± SD. The significant difference between experimental groups was performed with Student's *t*-tests. $p < 0.05$ was considered significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

RESULTS AND DISCUSSION

Enhancements of EGCG and Rutin on the Glucose Stimulated Insulin Secretion and Preservation of the Glucose Sensing Ability During the High Glucose Incubation. To investigate the metabolic effects of EGCG and Rutin in β cells, the impact on cellular insulin secretion was first investigated (chemical structures of EGCG and Rutin were presented in **Figure 1**a,b). Glucose stimulates intracellular ATP content, acting as a critical factor to regulate the insulin secretory machinery (*21*). The stimulation was observed on β cells after incubating in low or high glucose condition (3.3 or 33 mM, respectively) for 2 h, and consequently, EGCG and Rutin were found to elevate the intracellular ATP content (**Figure 1**c,d) and, correspondingly, it was also observed that the high glucose stimulated insulin secretions (**Figure 1**f). To examine whether EGCG and Rutin could still induce the secretory machinery of cells after long-term glucose stimulation, the β cells were incubated with high glucose for 48 h. In **Figure 1**e, the high dose (10 μM) of EGCG and Rutin both elevated the ATP content significantly ($p = 0.01$, 0.008 respectively); however, EGCG had less effect in the low dose treatment (0.1 μM, $p = 0.22$). The secretion of insulin was dramatically suppressed by

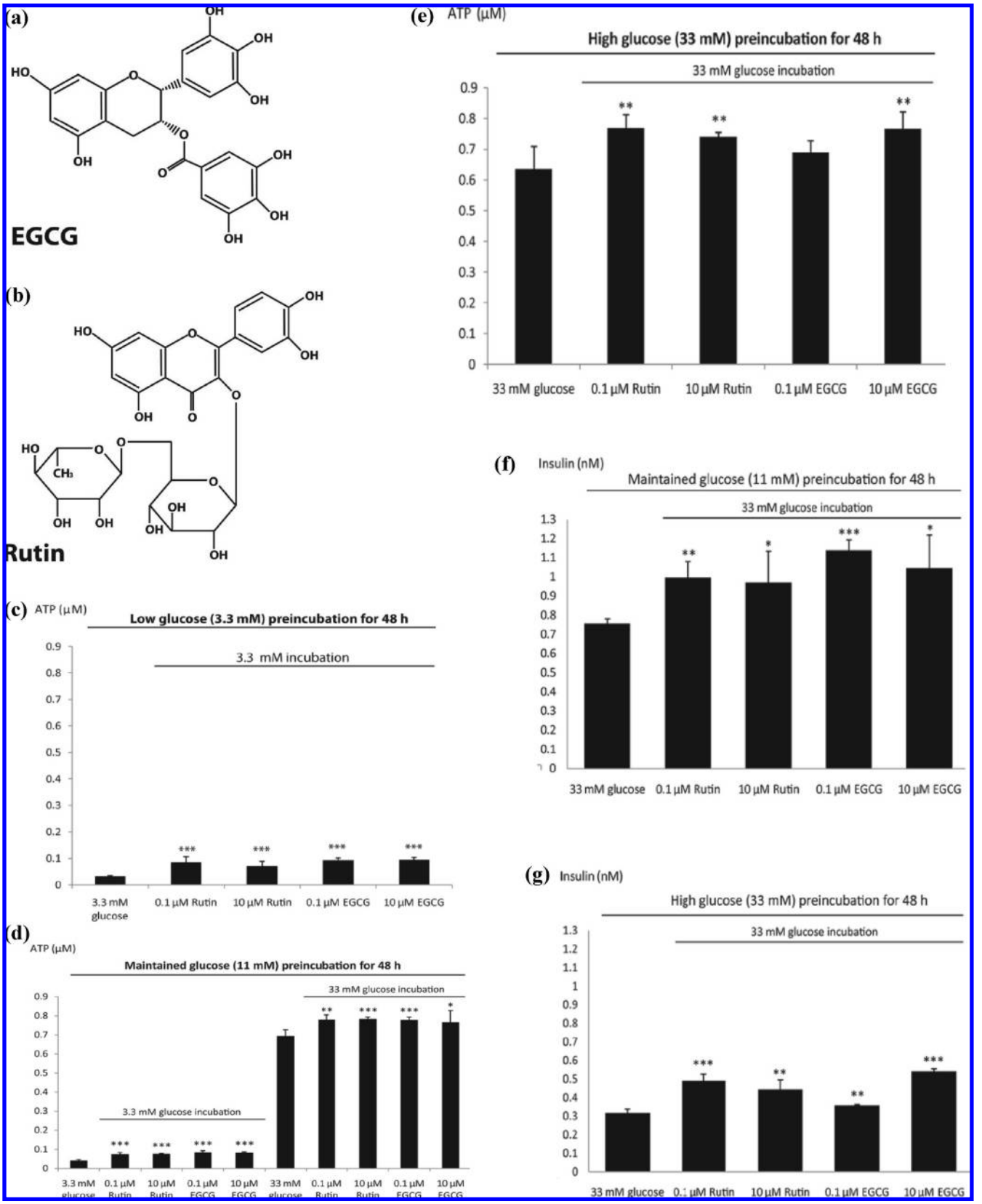

Figure 1. Enhancements of EGCG and Rutin on the glucose-stimulated insulin secretion and preservation of glucose sensing ability during high glucose incubation. (a, b) The chemical structures of EGCG and Rutin. (c)The cellular ATP contents were detected after the 48 h low glucose (3.3 mM) preincubation and 2 h stimulation by 3.3 mM glucose. Cells treated with EGCG/Rutin effectively increased the ATP contents compared to the control. (d)The cellular ATP contents were detected after the 48 h maintained glucose (11 mM) preincubation and 2 h stimulation of 3.3 or 33 mM glucose. Cells treated with EGCG/Rutin effectively increased the ATP contents compared to the control. (e) The cells were preincubated in 33 mM glucose contained medium for 48 h (chronic high glucose incubation), and then the medium was replaced with high glucose medium with or without EGCG/Rutin. The cellular ATP contents were detected over the next 2 h. (f) The insulin secretion was effectively increased in the action of EGCG/Rutin after the high glucose stimulation (2 h). (g) EGCG/Rutin could preserve the ability of glucose sensing after chronic high glucose incubation (48 h) and leads to the effective secretion of insulin in response to the demand. (*n* value at least equals to 3).

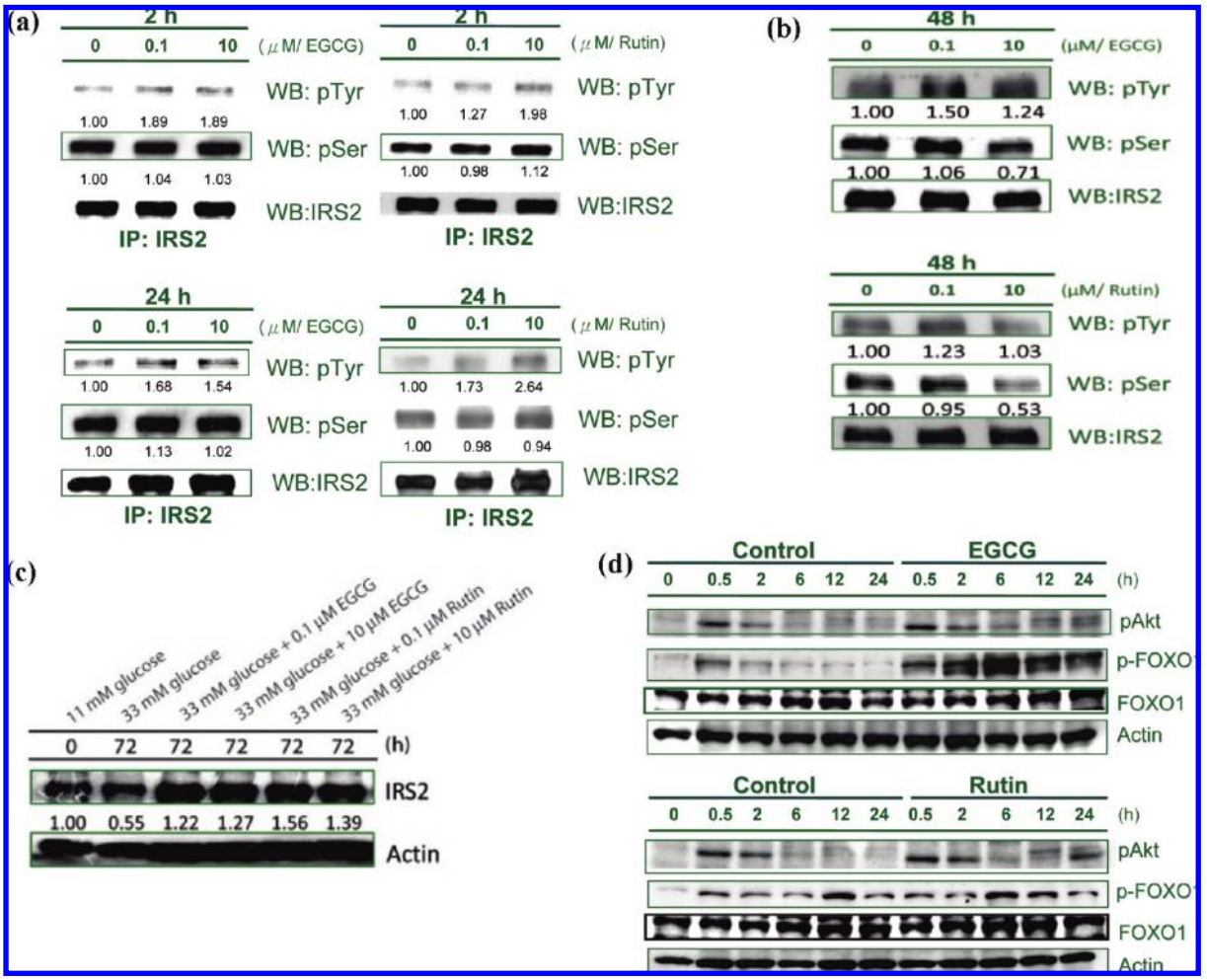

Figure 2. Effects on the stimulation of IRS2 signaling. (a, b) The IRS2 signaling activity was observed by tyrosine and serine phosphorylation through immunoprecipitation. Cells were incubated in high glucose condition treated with EGCG/Rutin. (a) The blotting analysis was performed and found that the enhancement of the IRS2 activation via Tyr phosphorylation after 2/24 h high glucose incubation through the treatment; the effective suppression from EGCG and Rutin of Ser phosphorylation did not display in this indicated time period. (b) Cells were incubated in chronic high glucose condition (48 h) treated with or without EGCG/Rutin. To compare with high glucose incubated control, decreased serine phosphorylated IRS2 were detected by treating with EGCG/Rutin; in contrast with the results of Ser phosphorylation, the Tyr phosphorylation was enhanced by treating with EGCG/Rutin. (c) Cells were incubated in 33 mM glucose condition and treated with 10 μM EGCG/Rutin. After the 72 h treatment, EGCG/Rutin promoted the expression of IRS2 correlated to the control in the high glucose incubation. (d) EGCG/Rutin effectively phosphorylated FoxO1 upon the IRS-2/Akt activation.

high glucose incubation; this suppression of insulin secretory machinery could be rescued through the EGCG or Rutin treatment (**Figure 1**g). The data suggested that EGCG and Rutin could help β cells to preserve the glucose sensing ability under long-term glucose exposure; in other words, EGCG and Rutin might be agents for the enhancement of pancreatic β cell secretory function when facing dysregulation by hyperglycemia.

Glucotoxicity is one crucially metabolic syndrome for the pathogenesis of diabetes mellitus, and how to balance the disturbance in metabolism has been a high concern. Previously, Tea polyphenol EGCG has been demonstrated to attenuate the hepatic insulin signaling blockade and show insulin mimicry (*6*, *12*); Rutin or buckwheat concentrate, instead, could reduce blood glucose in diabetic rats (*20*, *22*), with the result that EGCG and Rutin are considered as possible antidiabetes. The results of this current study revealed that EGCG and Rutin might enable the pancreatic β cells to sustain the adaptation from hyperglycemia and attenuate the glucotoxicity.

This study first implicated the action of EGCG and Rutin to increase the cellular energy (ATP) generation, which stimulates the insulin secretory machinery (**Figure 1**) and, moreover, preserves glucose sensitivity while maintaining the mitochondrial function. The preservation of insulin secretion allowed the cells to deal with chronic metabolic stress and, consequently, that might be the crucial factor for the manner in which EGCG and Rutin protect the integrity of β cells. Except for physical regulation, insulin also exerts the action in maintaining the β cell function through the autocrine effect; EGCG and Rutin could effectively increase the insulin secretion that occurs in response to glucose stimulation and restore the facility after chronic glucose exposure, which would directly enhance the activation of insulin/IRS signaling.

Effects on the Stimulation of IRS2 Signaling. Long-term exposure to glucose has been found to induce insulin resistance through reducing the IRS sensitivity and resulting in the blockade of insulin signaling (*23*). To determine the metabolic effects of EGCG and Rutin in β cells by protecting the activity of IRS2 signaling, cells were incubated in 33 mM glucose condition with or without the EGCG and Rutin treatment. The results showed that EGCG and Rutin stimulated the IRS2 signaling through enhancing tyrosine phosphorylation after 2 or 24 h under high glucose condition (**Figure 2**a), and then the stimulated effects

could be sustained until 48 h (**Figure 2**b). On the other hand, chronic hyperglycemia would elevate the oxidative stress, resulting in the phosphorylation of serine residues of IRS2, which are exhibited as a blocking signal; whereas, EGCG and Rutin could effectively reduce the level of serine phosphorylation of IRS2 after 48 h under high glucose incubation (**Figure 2**b). Meanwhile, the IRS2 expression was suppressed after a 72 h high glucose incubation, whereas EGCG and Rutin could reverse this suppression and enhance the expression level of IRS2 (**Figure 2**c). To summarize the effects of EGCG and Rutin in the expression and activation of IRS2, EGCG, and Rutin, the high dose treatment, especially, could enhance insulin signaling from overloaded metabolic demands and suppress insulin resistance from chronic glucotoxic effects.

The crucial factors are for developing and maintaining the β cell viability and function in which Akt, serine-threonine kinase, is another essential component in IRS signaling (*9*, *10*). To assess the action of EGCG and Rutin in the activation of Akt, the treated cells under high glucose condition were evaluated by Western blotting, which revealed that EGCG and Rutin both could preserve and extend the activation of Akt under high glucose condition (**Figure 2**d). The extended Akt activation efficiently accelerated the serine 256 phosphorylation of FoxO1, indicating that the nuclear exclusion of FoxO1 and, specifically, increased cytoplasmic phosphorylated FoxO1(pFoxO1) are meant to maintain the response of glucose stimulation and reduce the severity of glucolipotoxicity (*10*). In this event, EGCG and Rutin were found to have potential in the maintenance of insulin signaling, which could be the stringent issue in diabetes, and these data suggest that EGCG and Rutin in β cells might be acting as appropriate stimulators for insulin action.

Upregulation of the IRS2 expression level would be required for adaptation of β cell mass to counteract the high-fat diet-induced insulin resistance (*24*); IRS2 specific deletion in β cell, instead, could cause the impaired glucose-stimulated insulin secretion and glucose intolerance (*25*), which shows the involvement of IRS2 in delaying or prevent the onset of diabetes (*26*). Besides, the serine 307 phosphorylation of IRS1 has been indicated as a potential inhibitor that could block downstream signaling through the interference of tyrosine phosphorylation and the association of PI3K (*27*, *28*). Elevated oxidative stress would activate JNK (c-Jun NH2-terminal kinase) and PKC (protein kinase c), which have been reported to be involved in high glucose-induced serine phosphorylation of IRS1 (*29*); nevertheless, recent studies demonstrated that PKC might not be the kinase to phosphorylate the serine residues on IRS2 (*30*), but JNK and GSK3 (glycogen synthase kinase 3) could sequentially phosphorylate the serine 488 and 484 residues on IRS2 in hepatocytes (*31*), in which the serine phosphorylation would activate the proteasome degradation system to eliminate the IRS2 protein, which led to severe insulin resistance (**Figure 6**). The further investigation would be focused on whether EGCG and Rutin could regulate the ubiquitination level of IRS and the activity of GSK3 and JNK, which, combined with our previous study, attenuates the insulin signaling blockade by EGCG in high glucose induced hepatocytes (*6*), providing the direct evidence of EGCG and Rutin for resolving the insulin resistance in endocrine system.

Enhancement of PDX-1 Expression. PDX-1, one key element on β cells, regulates the transcription of insulin, GLUT-2 (glucose transporter 2), GK (glucokinase), and Nkx 6.1, and is involved in β cell differentiation and functional maintenance (*32*). EGCG and Rutin effectively reversed the suppression of PDX-1 after long-term high glucose incubation (**Figure 3**a,b). Moreover, the immunocytochemical images also revealed that EGCG and Rutin could maintain the expression of PDX-1 after a 72 h high glucose incubation (**Figure 3**c). The high glucose control (33 mM) diminished the expression of PDX-1 in the cytoplasm and nucleus, which corresponded to the result presented in Western blotting; on the contrary, cells incubated in low maintained glucose (11 mM) condition expressed no suppressive effect on the PDX-1 expression in cytoplasm after 72 h. And the treatment of EGCG and Rutin could remarkably enhance the expression of PDX-1 in cytoplasm and nuclei, presenting homogeneous PDX-1 expressions within cells as demonstrated by the localization of fluorescence (**Figure 3**c). The deficiency of PDX-1 manifests MODY4 (maturity-onset diabetes of the young), one type of genetic deficient diabetes. The impact of PDX-1 expression on the regulation from the pancreas even evolves to whole body metabolism. Hyperglycemia or hyperlipidemia could interfere with insulin signaling and the activity of PDX-1, regarded as factors for contributing to the dysfunction of β cells, while EGCG and Rutin could reverse the interference of glucotoxicity, indicating the possibility for retaining the integrity of cellular function. These results imply that the EGCG and Rutin might have the protective ability for β cells against massive metabolic stress.

The activity of Akt is essential for β cells in the insulin secretion, cell survival, and proliferation (*9*); the prolonged activated Akt was observed after the treatment of EGCG and Rutin (**Figure 2**d), describing that the treatment reversed the chronic high glucose-induced inactivation and enhanced the phosphorylation of FoxO1, facilitating the transcription of PDX-1, which in previous studies has been shown to be required for insulin action and β cell function (*33*, *34*). PDX-1 could critically exert the manner in which the health of β cells is maintained (*35*, *36*), and at the same time the concert of PDX-1 with other significant transcription factors, MafA and NeuroD1, could be enhanced by the action of EGCG and Rutin. Previous reports have shown that overnutrition would inhibit insulin gene expression caused from the alteration of PDX-1 localization and the concert activity (*37*, *38*), indicating the significance of PDX-1 in β cells and, furthermore, that might be suggested to exert the preservation of the insulin secretory machinery from the effects of EGCG and Rutin. PDX-1 could conduct the transcription not only in insulin but in other significant proteins, GK and GLUT-2, involved in glucose metabolism. GK has been recognized as a glucose sensor and has indicated the requirement for β cell compensation (*24*) and the mitochondrial function (*39*).

Suppression of Glucolipotoxicity by EGCG and Rutin via AMPK Activation. Hyperglycemia could upregulate the lipogenesis, causing ectopic lipid deposition and even result in the dysfunction and reduced viability in nonadipocytes; especially, that which is prone to occur in the most lipid-laden cell, β cell (*40*). To assay whether EGCG and Rutin exert the manner in which the cellular lipid metabolism is regulated, the activation of AMPK was first evaluated, by Western blotting, as a cellular energy sensor that is critically involved in lipid metabolism. Indeed, EGCG and Rutin rapidly increased the activity of AMPK through threonine 172 phosphorylation (**Figure 4**b,c) and maintained the enhanced activity after a 72 h high glucose incubation. Moreover, AMPK might be a suppressor of ROS (reactive oxygen species), leading to a concern with the activity of AMPK and the functional failure of β cells (*41*). As the results indicated, EGCG effectively suppressed the generation of ROS (**Figure 4**a (i, ii)) but Rutin had less effect (**Figure 4**a (iii, iv)), which could be related to the exertion on the activation of AMPK. The activity of AMPK could be regulated by upstream factors, LKB1, while EGCG and Rutin could increase the expression of LKB1 (**Figure 4**d,e), suggesting the mechanism for stimulating AMPK might counteract the glucolipotoxicity induced by the high glucose condition.

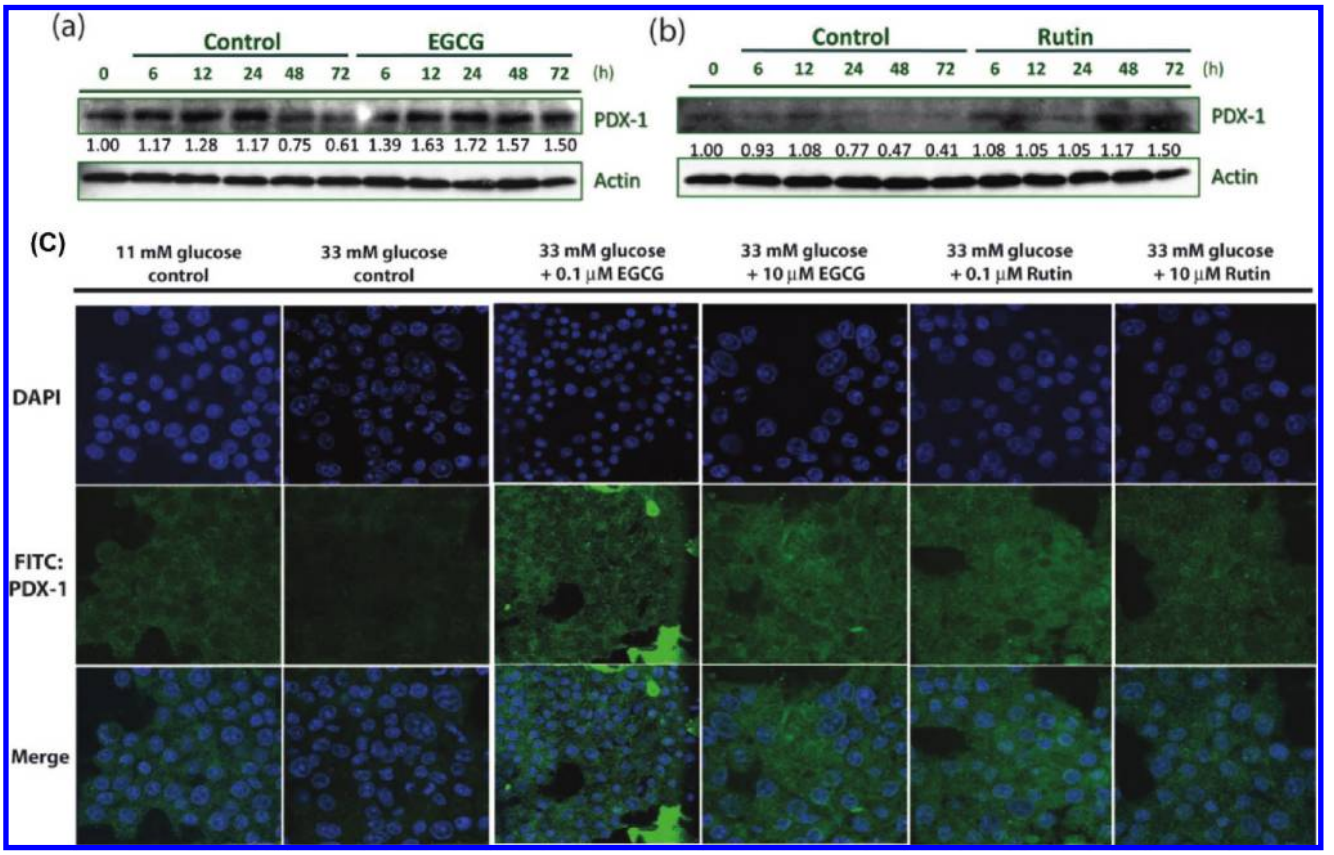

Figure 3. Alteration of PDX-1 nuclear translocation and expression. (a, b) Under high glucose incubation, 10 μM EGCG (a) and 10 μM Rutin (b) the expression of PDX-1 was substantially increased; however, high glucose incubated control decreased the expression level after chronic high glucose exposure. (c) Cells were incubated in 33 mM glucose condition for 72 h. To compare with high glucose control, EGCG/Rutin promoted PDX-1 to process the nuclear translocation (scale bar = 25 μm), as demonstrated by Leica TCS SP5 confocal microscopy.

Currently, several key targets of AMPK in lipid signaling, including ACC (acetyl-CoA carboxylase), FAS (fatty acid synthase), MCD (malonyl-CoA decarboxylase), and SREBP1 (sterol-regulatory-element-binding transcription factor 1) (*15*), have been linked to the manner in which β cells fail. EGCG and Rutin demonstrated the suppressive effect of FAS expression and ACC inhibition through the serine 79 phosphorylation (**Figure 4**d,e). The suppression of intracellular lipogenesis, however, was not directly manifested yet; to survey the action of EGCG and Rutin, oil red O staining was performed to confirm the level of lipid accumulation within the β cells. It appeared that cells under chronic high glucose incubation (5 day incubation in 33 mM glucose) had increased accumulated lipid droplets by 43.3% in 33 mM glucose treated cells as compared to the maintenance condition (11 mM; **Figure 4**f), and EGCG and Rutin could inhibit the ectopic lipid deposition dose-dependently. The intracellular lipid content correlated to insulin resistance in liver and muscle, while that also correlated to cell dysfunction and even led to apoptosis in pancreatic β cells, with the result that EGCG and Rutin might display the possibility for defending the toxicity that occurs from chronic metabolic stress.

Activated Akt is directly involved in β cell growth and survival and regulating p21, Cyclin D1, and Bax (*10*, *42*, *43*), and the results show the enhanced effects of EGCG and Rutin in cell viability and enabled to attenuate the long time exposure of glucose, which could be mediated by regulating the cell cycle related proteins. Bcl-2, antiapoptotic protein, in these observations, as well as previous studies, was demonstrated to preserve the viability, under the lipotoxic condition (*44*), the action of EGCG and Rutin to suppress the glucolipotoxicity and effectively elevate the expression of Bcl-2. To concern the cell cycle regulated proteins in the effects of EGCG and Rutin, another critical element, mitochondria, required for cell viability, has also been deliberated. Mitochondria are a cellular energy factory to support cells for survival, and the dysfunction of mitochondria has been proven to be involved in the cause of β cell failure and the development of diabetes (*45*, *46*). Previous reports have indicated that EGCG could increase antioxidative enzyme activity and preserve the integrity of mitochondria when dealing with the deleterious effects of lipids (*47*, *48*); the current investigations demonstrated that EGCG and Rutin could either increase the mass of mitochondria or raise the mitochondrial membrane potential after chronic glucose exposure, suggesting that EGCG and Rutin might mediate the protection of mitochondria for β cells against glucotoxic effects.

Maintenance of Cell Mass against Chronic Glucotoxicity. Hyperglycemic condition could lead to glucose-induced deterioration of cell mass progressing in the pathogenesis of diabetes, accompanied with decompensation of pancreatic β cells (*3*). The results from the MTT assay demonstrated that EGCG and Rutin effectively preserved the cell mass and the proliferation ability after 120 h (**Figure 5**a (i, ii)), suggesting that the treatment might benefit the cells to attenuate the chronic metabolic stress. Additionally, the low dose treatment of Rutin compared to the high dose treatment demonstrated a more vigorous effect for cell proliferation after a 72 h high glucose incubation, suggesting it could be caused from the effects on the enhancement of IRS2 expression through suppression of IRS2 serine phosphorylation (**Figure 2**b,c). To further evaluate the benefit for cells, the cellular energy factory, mitochondria, was considered because mitochondrial dysfunction

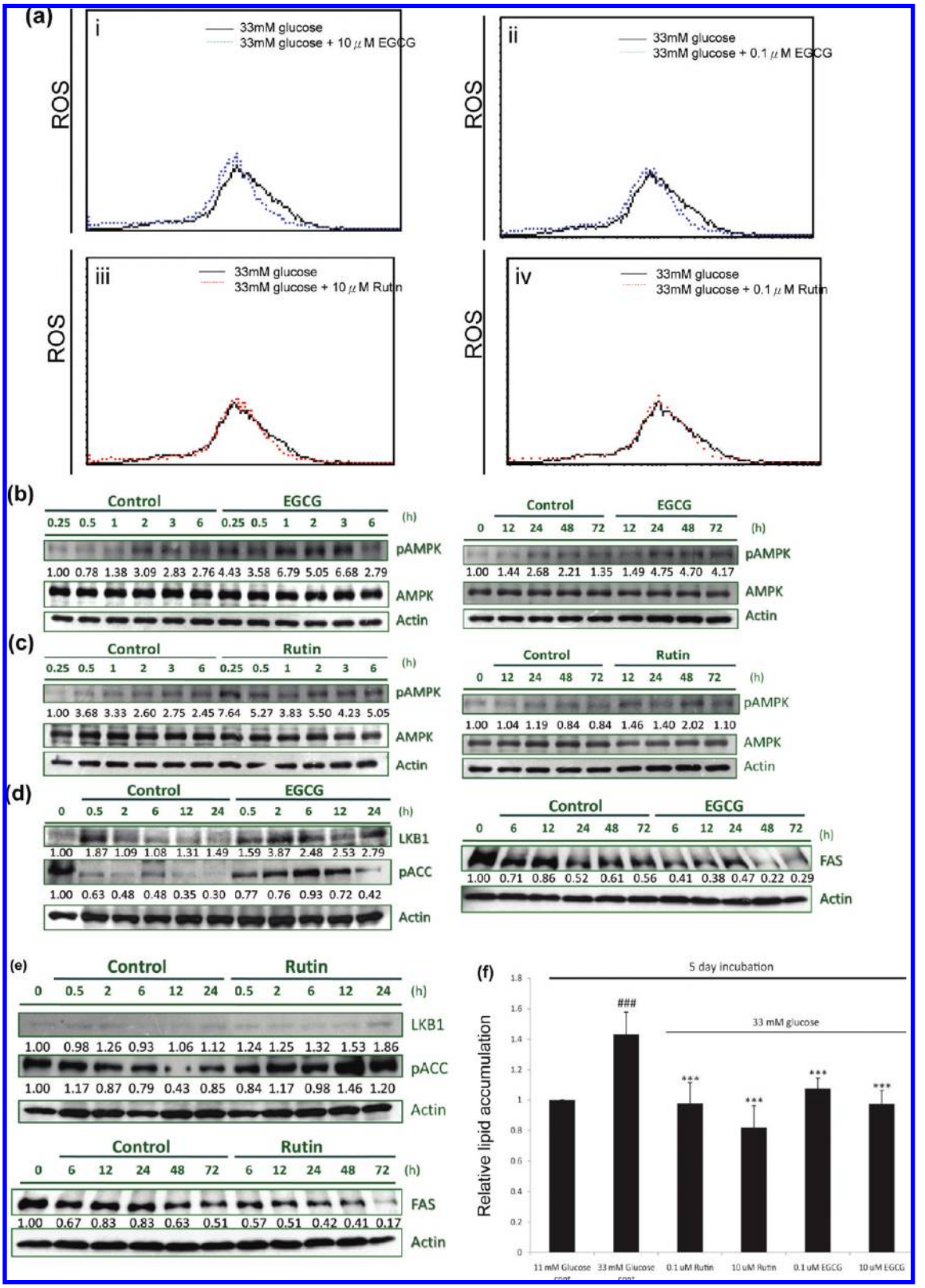

Figure 4. Suppression of EGCG and Rutin in glucolipotoxicity via AMPK activation. (a) (i, ii) Cells with EGCG were assayed by flow cytometry. EGCG reduced cellular ROS generation presented by the left-shifted dot line under 33 mM glucose incubation; instead, cells with Rutin had less effect on reducing ROS generation (iii, iv) (Solid line = 33 mM glucose control cells, dot line = ECGC- or Rutin-treated cells). (b, c) Cells were incubated in high glucose condition and treated with 10 μM EGCG/Rutin. The high glucose incubation suppressed AMPK activity and increased the FAS expression with incubation period. EGCG/Rutin reversed the AMPK activity in the high glucose incubation and led to the suppression of the FAS expression. (d, e) ECGC/Rutin activated AMPK by the mediation of LKB1 and led to the suppression of the activity of ACC via phosphorylation. (f) After the chronic high glucose incubation (5 days/ 33 mM glucose), the cells accumulated lipid droplets by the metabolic stress. However, cells treated with EGCG/Rutin effectively reduced the lipid accumulation and declined the level to the maintenance glucose control (11 mM glucose; *n* value at least equals to 6; #the significance of 33 mM glucose control compared with 11 mM glucose control; *the significance of 33 mM glucose treated with EGCG/Rutin compared with 33 mM glucose control).

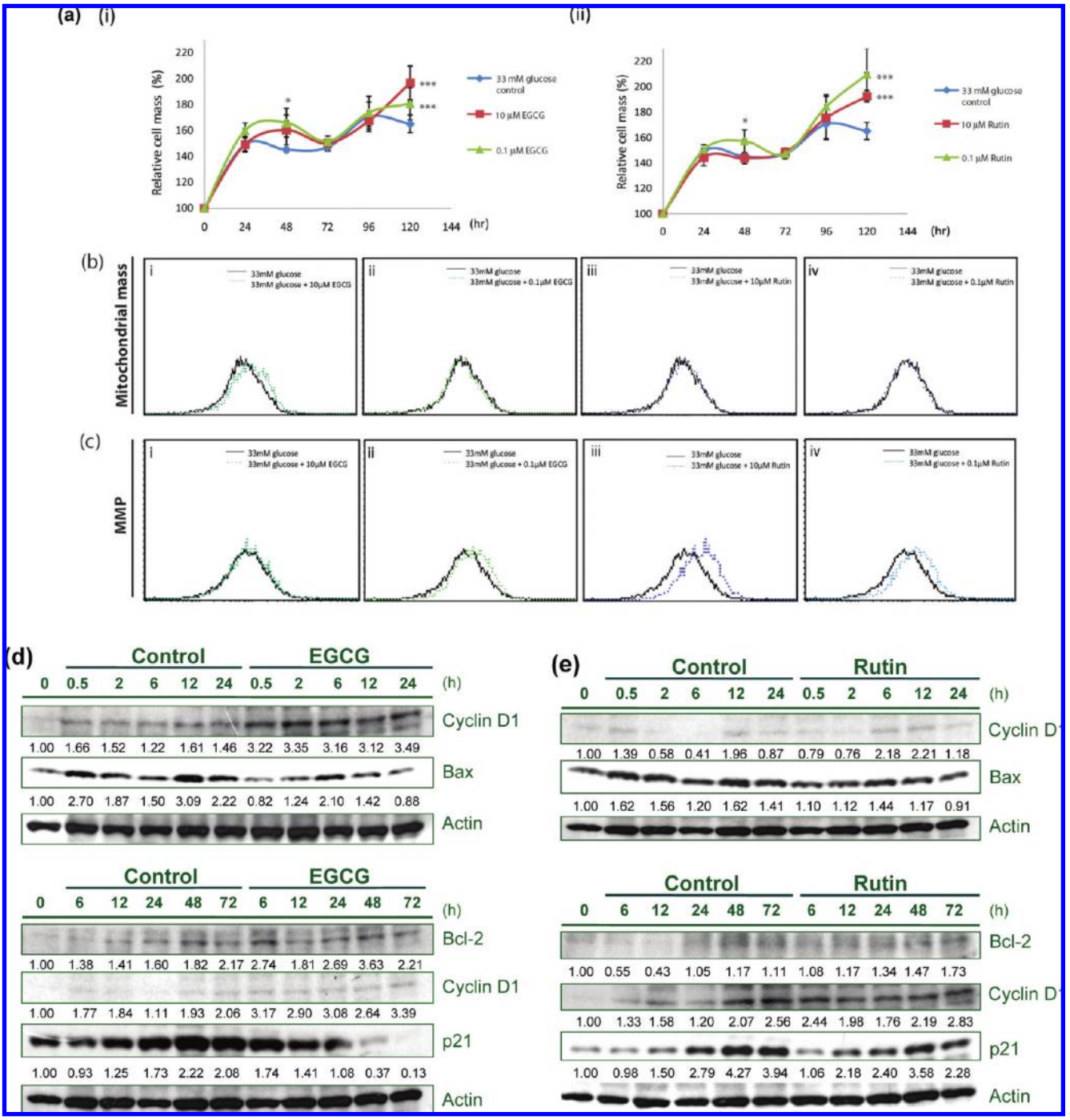

Figure 5. Maintenance of cell mass against chronic hyperglycemic incubation. (a) Cells were incubated in chronic high glucose condition (33 mM) with or without EGCG (i)/Rutin (ii) treatment. Cell mass was performed by MTT assay, revealing that EGCG/Rutin effectively preserved and increased cell mass after the high glucose incubation (120 h). (b, c) Cells were incubated in 33 mM glucose condition for 96 h, treated with or without EGCG/Rutin, and then stained with Mitotracker or DiO6 for detecting the mass and mitochondrial membrane potential, respectively. (b) High dose EGCG (i) treated cells could effectively increase the mass of mitochondria, presented by the right-shifted dot line; however, the treatments of low dose EGCG (ii) and Rutin (iii, iv) had no distinct effects. (c) Instead, low dose EGCG (ii) and Rutin (iii, iv) treatment expressed the effect on evoking the mitochondrial membrane potential (solid line = 33 mM glucose control cells, dotted line = EGCG- or Rutin-treated cells). (d, e) EGCG/Rutin suppressed the cellular p21 and Bax expression in high glucose incubation. On the other hand, the elevation of Cyclin D1 and the antiapoptotic protein Bcl-2 expression had also been found.

was correlated with the initiation of diabetes mellitus and apoptosis (*45*, *46*). Flow cytometry analysis demonstrated the increased intention of the mitotracker fluorescence from high dose EGCG treatment (**Figure 5**b (i)), indicating the mass of mitochondria were efficiently multiplied after 96 h at high glucose incubation, while Rutin and low dose EGCG treatment manifested the effect not only on the mass but also augmentation of the mitochondrial membrane potential (**Figure 5**c (ii, iii, iv)). The data illustrated that EGCG and Rutin enhanced the mass or functional integrity of mitochondria, implicating the reinforcements of EGCG and Rutin for the mitochondrial machinery under high glucose stimulation, which might facilitate to resolve the overloaded stress.

To address the mechanism of EGCG and Rutin in the preservation of cell viability, cell cycle related proteins were

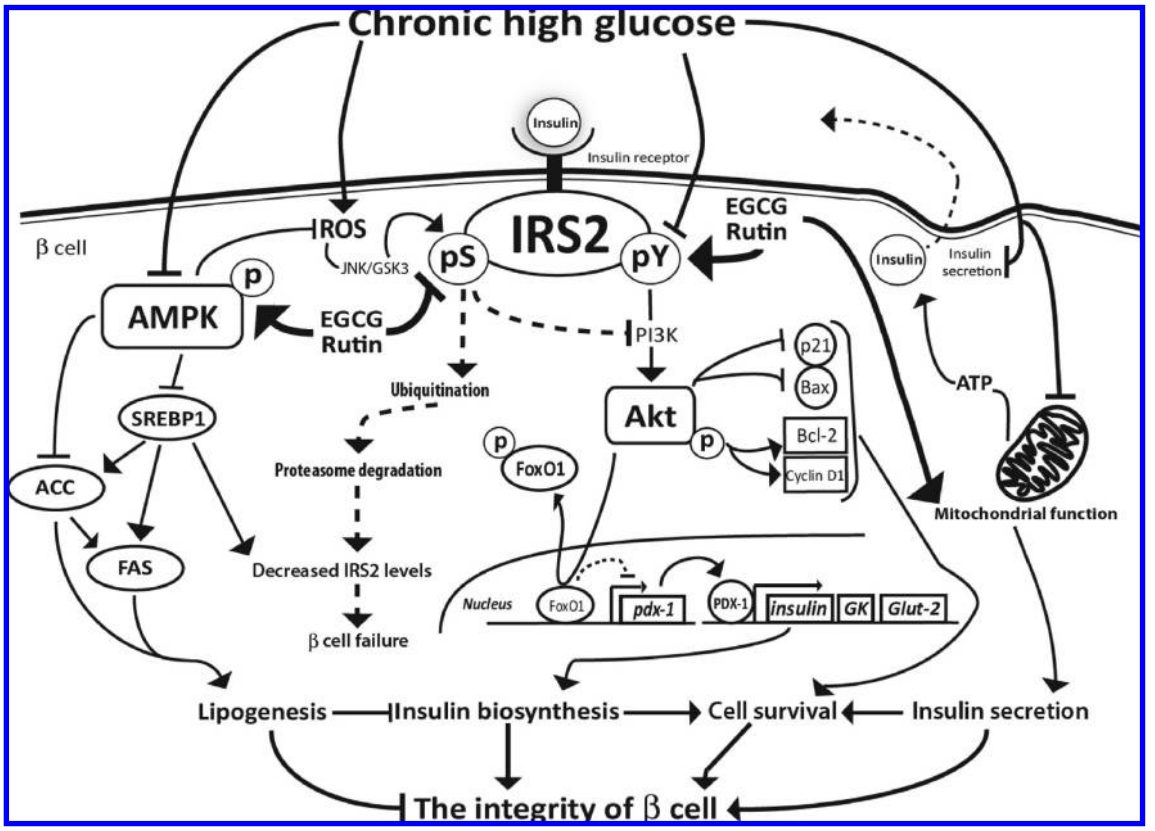

Figure 6. Attenuation of glucotoxicity through modulating IRS2 signalings. Chronic high glucose exposure would directly increase intracellular ROS generation and deteriorate mitochondrial function to uncouple with ATP generation, impairing the glucose stimulated insulin secretion. Suppression of IRS2 signaling could be induced by serine phosphorylation caused from JNK and GSK3 activation, which would disrupt the tyrosine phosphorylation and the recruitment of PI3K to activate downstream signaling, Akt, FoxO1, and PDX-1. Akt displays multiple roles in pancreatic β cells, regulating cell proliferation through Cyclin D1 and Bcl-2 and suppressing cell apoptosis through p21 and Bax; otherwise, Akt phosphorylates FoxO1 to sequester it from the nucleus, facilitating the transcription of PDX-1, which is critical for insulin synthesis and cell survival. Chronic high glucose exposure suppressed AMPK activity and increased lipogenesis through activating lipogenic enzymes, ACC and FAS. Lipid accumulated in β cells would interfere with the normal cellular function; SREBP1, a crucial transcription factor in lipid metabolism, would suppress IRS2 and PDX-1 expression and increase intracellular lipogenesis. EGCG and Rutin could reverse the IRS2 and AMPK suppression from high glucose induction, protecting the integrity of pancreatic β cells to deal with glucotoxicity. The dotted line presented the further investigation of whether EGCG and Rutin could attenuate the IRS2 degradation through regulating ubiquitination.

investigated. Previously it was observed that EGCG and Rutin enhanced the activation of Akt (**Figure 2**d); with concerting with insulin signaling, Akt could critically participate in the processes of proliferation through the interaction with Cyclin D1, p21, Bcl-2, and Bax. To evaluate the effects of EGCG and Rutin on cell cycle regulation, the results from immunoblotting showed that EGCG and Rutin effectively increased the expression of Cyclin D1 and Bcl-2 (**Figure 5**d,e), the vital factors for β cell viability. High glucose stimulation could increase the expression of Bax and p21, depending on the duration of incubation; meanwhile, EGCG and Rutin reversed the effects, causing the suppression of Bax and p21 (**Figure 5**d,e). Stimulation of high glucose to β cells would decrease the viability through the regulators of proliferation, with the result that the data suggested the action of EGCG and Rutin not only preserve the cellular function but enhance the survival signal against the glucotoxicity.

Chronic high glucose exposure could gradually stress pancreatic β cells to compensate the overloaded metabolism and then that could induce the development of glucotoxicity. According to the current data presented, the investigated mechanisms of EGCG and Rutin in pancreatic β cells are summarized in **Figure 6**. EGCG and Rutin could attenuate the glucotoxic effects through activating IRS2 and AMPK signaling; the enhancement of insulin signaling to increase the active tyrosine phosphorylation signal, but otherwise to decrease the negative serine phosphorylation signal, preserves the insulin sensitivity and facilitates the downstream signaling, Akt, FoxO1, and PDX-1. The activation of AMPK attenuated lipogenesis and suppressed ROS generation, reducing the glucolipotoxicity and mitochondrial function. Consequently, EGCG and Rutin benefit the pancreatic β cells to sustain the metabolic stress, revealing the possibility of preventing the progression of type 2 diabetes.

AMPK activity is considered to not only be involved in fuel sensing, but to be linked with insulin resistance and the cellular lipogenesis (*15*). Developed antidiabetic drugs, metformin and TZDs (Thiazolidinediones), could activate AMPK, treating for insulin resistance and disorders associated with the metabolic syndrome. These findings described that EGCG and Rutin could increase AMPK activity through LKB1 and then suppress ROS generation (**Figure 6**). Compared with recent reports that indicated the similar effects that activated AMPK could suppress ROS generation, inhibiting palmitate-induced apoptosis in endothelial cells (*41*), and resveratrol, from red wine polyphenol, which could reduce the ROS-induced toxicity through AMPK in cardiac muscle cells (*49*); rather, these current findings inferred that the tea polyphenol and buckwheat flavonoid could exert the manner of AMPK in β cells.

This study highlights the novel function of EGCG and Rutin in rat pancreatic β cells, integrating the molecular effects of glucose and lipid metabolism. Consequently, these data suggested that

EGCG and Rutin might be potential agents for the attenuation of type 2 diabetes through the protection of pancreatic β cells.

ABBREVIATION USED

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; EGCG, (−)-epigallocatechin-3-gallate; FAS, fatty acid synthase; FoxO1, forkhead-O transcription factor 1; GSIS, glucose stimulated insulin secretion; MMP, mitochondrial membrane potential; PDX-1, pancreas-duodenum homeobox-1; ROS, reactive oxygen species; SREBP1, sterol-regulatory-element-binding transcription factor 1; JNK, c-Jun NH2-terminal kinase; PKC, protein kinase c; GSK3, glycogen synthase kinase 3.

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