The Effect of SIN1 and Microtubules on Insulin Induced PKC ζ Activation

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
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Background: Protein kinase C zeta (PKC ζ) plays an important role in insulin induced glycometabolism and insulin receptor (IR) associated signaling pathways. The full activation of PKC ζ depends on its translocation from cytosol to membrane and phosphorylation at Thr410. However, the mechanism of PKC ζ activation remains elusive. In this study, the effect of SIN1 and microtubules on insulin-induced PKC ζ activation was investigated.

Material/Methods: HepG2 cells were stimulated with insulin for co-immunoprecipation (co-IP) assay. The immunocomplex was captured by using anti-PKC ζ, anti-SIN1 or anti-FLAG antibodies and was subjected to western blotting analysis for detecting PKC ζ, SIN1, and β-tubulin protein expression level. The cells were intervened by small interfering RNA (siRNA) that targeted exon regions of SIN1. Then the glucose uptake ratio after cells were stimulated by insulin was measured. The PKC ζ insulin receptor levels in the membranes were analyzed. Cells stained with anti-PKC ζ, anti-SIN1 antibodies and probed with molecular probes were observed by immunofluorescence confocal microscopy.

Results: SIN1 interacted and co-located with PKC ζ by pleckstrin homology (PH) domain. Downregulation of SIN1 severely impaired PKC ζ translocation and phosphorylation induced by insulin. PKC ζ co-immunoprecipitated with β-tubulin at different intervals upon insulin stimulus, and the activation of PKC ζ was affected by paclitaxel and nocodazole.

Conclusions: PKC ζ translocated from cytosol to membrane depending on SIN1, which suggested that PKC ζ may be activated directly by PI3K and the reaction probably carried out on microtubules in HepG2 cells.

MeSH Keywords: Insulin • Microtubules • Protein Kinase C

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Background

Insulin resistance is a ubiquitous pathological process in patients with type 2 diabetes; the term refers to insulin target organs (e.g., muscle and liver) that are not sensitive to insulin stimulus, which leads to a disorder in glucose uptake and utilization [1]. As the incidence of type 2 diabetes is increasing rapidly [2,3], exploring the mechanism of insulin resistance becomes important. Atypical protein kinase C (PKC) isoform PKC zeta (PKC ζ) has been reported to be activated in insulin-stimulated glucose transport through the PI3K/PKC ζ pathway [4–7]. Inhibition of the PKC ζ enzyme inhibits insulin-stimulated glucose transport while activation of PKC ζ increases glucose transport and ameliorates hepatic steatosis in obese diabetic mice [8,9]. One study found that PI3K subunit p85 transferred from cytosol to the cytomembrane within one minute under 10^{-7} mol/L insulin stimulus in rat fibroblasts in which insulin receptor (IR) was highly expressed, while PKC ζ transferred from cytosol to the cytomembrane within one to 10 minutes [10], which suggested that PI3K activation occurred prior to PKC ζ, and PI3K may regulate PKC ζ directly in the cytomembrane. In a resting state, inactive PKC ζ locates in the cytoplasm, while it translocates to the cell membrane and becomes active upon stimulus [11]. However, how PKC ζ translocates from cytosol to the cytomembrane remains unclear.

The mTOR complex 2 (mTORC2) also plays a pivotal role in cell metabolism, growth, proliferation, and survival [12,13]. mTORC2 deficient hepatocytes display loss of glucokinase activity in the liver, which leads to constitutive gluconeogenesis, and impair glycolysis and lipogenesis [14]. mTORC2 regulates glycometabolism through downstream substrates and an abundance of evidence demonstrates that mTORC2 phosphorylates and activates AGC kinase family members, including the PKC family [15,16]. SIN1, as a unique subunit of mTORC2, is important for the complex assembly as well as function. SIN1 is conserved in all eukaryotic species and has a tissue expression similar to that of mTOR [17]. It has a conserved region in the middle of the sequence, a Ras-binding domain and a C-terminal pleckstrin homology (PH) domain [18]. According to previous reports, the SIN1 PH domain connects mTORC2 to PI3K [19] and is involved in membrane targeting of many signal molecules [20]. Yao et al. have shown that the PH domain of Bruton tyrosine kinase could interact with PKC, including both Ca^{2+}-dependent (α, βI, and βII) and Ca^{2+}-independent PKC isoforms (ε and ζ) [21]. Taken together, the hypothesis is proposed that SIN1 interacts with PKC ζ via its PH domain and contributes to its translocation from cytosol to membrane.

Material and Methods

Plasmid construction

The full-length pCMV-SIN1-FLAG (nucleotide ID of SIN1: NM_001006617.1) was constructed by PCR cloning into pCMV5; the C-terminal truncated (ΔPH) SIN1 (1~382) was constructed using pCMV-SIN1-FLAG as template.

Cell culture and transfection

HepG2 cells were obtained from the American Type Culture Collection, where they were characterized by DNA profiling. HepG2 cells were cultured in DMEM (Gibco, 25 mM glucose) with 10% FBS (HyClone) at 37°C in 5% CO_2. The cell lines were passaged for less than six months in this study. Lipofectamine 2000 (Invitrogen) was used for transiently transfection according to the manufacturer's instructions.

siRNA interfering

Three stealth siRNAs were designed to target different exon regions of human SIN1 with the sequences as follows: (#1: 5'-CAGUCACGCAAUAUUUAAAUUUUUAA AUAUUGCGUGACUGTT-3';#2: 5'-GGGAAGCAGUCGAUAUUA UUUAUAAUAUCGACUGCUUCCCTT-3';#3: 5'-CUGCUACCGU CAAUGAAAUUAUUUCA UUGACGGUAGCAGTT-3';) and a scrambled (Scr) siRNA were synthesized by Genepharma. The molar concentration of SIN1 and Scr were the same in this study.

Co-immunoprecipitation (co-IP) assay

Co-IP was performed as previously described [22]. Briefly, HepG2 cells were seeded in 10 cm dishes and grown to 80% to 90% confluence. After 12 hours of non-serum starvation, cells were either left unstimulated or stimulated with 100 nmol/L insulin (Novolin R, Novo Nordisk) for 10 minutes, lastly for 30 minutes for co-IP with β-tubulin (Affinity, T0023). After washing three times with PBS, cells were lysed in 1 mL of ice-cold cell lysis buffer (0.3% CHAPS, 1.5 mmol/L Na_3VO_4, 40 mmol/L HEPES, 120 mmol/L NaCl, 10 mmol/L pyrophosphate, 10 mmol/L glycerophosphate, 50 mmol/L NaF, 1 mmol/L EDTA, and complete protease inhibitor cocktail (Roche), pH 7.5) on ice for 30 minutes. After centrifugation at 12,000× g for 10 minutes, supernatants were removed and precleared by adding 50 μL of protein G (Novex) for one hour. Supernatants were immunoprecipitated by using anti-PKC ζ (Santa Cruz, Sc-216), anti-SIN1 (Millipore, #05-1044) or anti-FLAG antibodies (Proteintech). The immunocomplex was captured by adding 50 μL of protein G and subjected to western blotting analysis (anti-SIN1 antibody dilute rate 1: 400, anti-PKC ζ antibody 1: 1,000, and anti-tubulin β antibody 1: 3,000).

Glucose uptake

Hepatocytes glucose uptake assay was done according to "National operation regulations for clinical examination" in China. At first, cells were intervened by DMEM containing 0.6 mmol/L palmitic acid for 12 hours. Then supernatant was collected at prescribed times and supernatant glucose concentrations were measured via Glucose Oxidase Method [23,24]. The supernatant for Scr cells and siRNA cells in 6 cm dishes were removed and changed with phenol red-free DMEM (Gibco) with 10% FBS. Cells were either left unstimulated or stimulated with 100 nmol/L insulin (Sigma) for 24 hours. After intervention, the cell supernatant was collected in a tube, centrifugation at 12,000×g for 10 minutes; then 5 uL of supernatant was added into 195 uL of working solution (Glucose Oxidase Method Kit; Applygen, Beijing), 37°C for 20 minutes; the OD value was measured at 550 nm wave length. The supernatant glucose concentrate was calculated as follows: supernatant glucose concentrate (mmol/L)=standard concentration×(unknown OD–blank OD)/(standard OD–blank OD)

Membrane fraction isolation

A protease inhibitor cocktail and phosphatase inhibitor cocktail were added to the cell lysis buffer before protein extraction. To isolate membrane fraction, cells were resuspended in 500 μL of subcellular fractionation buffer (250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L EDTA, and protein inhibitor cocktail) at 4°C and lysed through a 25-gauge needle 10 times using a 1 mL syringe. Lysates were centrifuged at 1,000×g for 10 minutes to remove nuclear pellets, and supernatants were centrifuged again at 100,000×g for one hour. The supernatant was the cytosol fraction. The pellet was the membrane fraction and it was washed and resuspended in the lysis buffer with 10% glycerol and 0.2% SDS for further analysis. Antibodies against insulin receptor were from Affinity Biosciences (AF6099, dilute rate 1: 600).

Immunofluorescence confocal microscopy

HepG2 cells were plated in 12-well plates containing sterile coverslips, allowed for 12 hours growth, and starved in serum-free DMEM for at least 12 hours. After stimulation with 100 nmol/L insulin for 10 minutes, cells were fixed with 4% paraformaldehyde, quenched with 50 mmol/L NH_4Cl, permeabilized in 0.2% Triton X-100 in PBS, and blocked in 3% bovine serum albumin. Cells were stained with anti-PKC ζ (1: 50) and anti-SIN1 (1: 20) antibodies (β-tubulin 1: 200) and probed with either an Alexa Fluor 568 (Molecular Probes, A-11011) or an Alexa Fluor 488 (Molecular Probes, A-11001) conjugated secondary antibody (1: 200). Coverslips were mounted and cells visualized with confocal laser scanning microscopy (Leica TCS SP5).

The rest of reagents

Paclitaxel (PTX) was obtained from DaLian Meilun Biotech Co., Ltd. (Dalian, China) and nocodazole (NDZ) were purchased from Sigma-Aldrich (Merck, USA); anti p-cofilin antibody from Santa Cruz (Sc-12912-R, dilute rate 1: 1,000); and antibodies to phospho-PKC ζ from Cell Signaling Technology (#9378, dilute rate 1: 1,000).

Statistical analysis

Results were expressed as mean ±SEM. Student-Newman-Keuls were used to examine differences in response to treatments between groups. Statistical evaluation was performed using SPSS13.0. The significance level was set at $p < 0.05$ (.), $p < 0.001$ (**), and "NS" was no significance. All assays were repeated at least three times and protein concentrations were measured by BCA protein assay.

Results

SIN1 downregulation impaired glucose uptake in HepG2 cells

To downregulate SIN1, all of the three stealth sequences severely decreased the expression of SIN1 in HepG2 cells, whereas the scrambled had no effect (Figure 1A). SIN1 downregulation decreased the ratio of glucose uptake in HepG2 cells in both 100 nmol/L insulin stimulus cells and non-insulin stimulus cells, approximately 27% and 20% with or without insulin stimulus, respectively (Figure 1B). Since the three siRNAs had similar phenotypes, the results of #3 were presented as the representative (siSIN1).

SIN1 associated with PKC ζ and co-localization with PKC ζ via its PH domain

The interaction between SIN1 and PKC ζ was examined by co-IP assays. As shown in Figure 2A and 2B, SIN1 and PKC ζ coprecipitated with each other *in vitro*, suggesting SIN1 may bind PKC ζ to form a complex. Next, we investigated the possibility that SIN1 interacts with PKC ζ in hepatocytes. Confocal microscopy analysis showed that both SIN1 and PKC ζ were distributed in the cytosol of resting cells, consistent with previous reports. On stimulation with 100 nmol/L insulin for 10 minutes, both PKC ζ and SIN1 proteins were enriched along the plasma membrane, suggesting activation of both proteins (Figure 2C), and SIN1 colocalized with PKC ζ on the plasma membrane. Taken together, the results suggested that SIN1 may bind PKC ζ to form a stable complex. In order to find the binding region of SIN1 with PKC ζ, two plasmids expressing wild SIN1 and C-terminal truncated SIN1 (1~382), respectively,
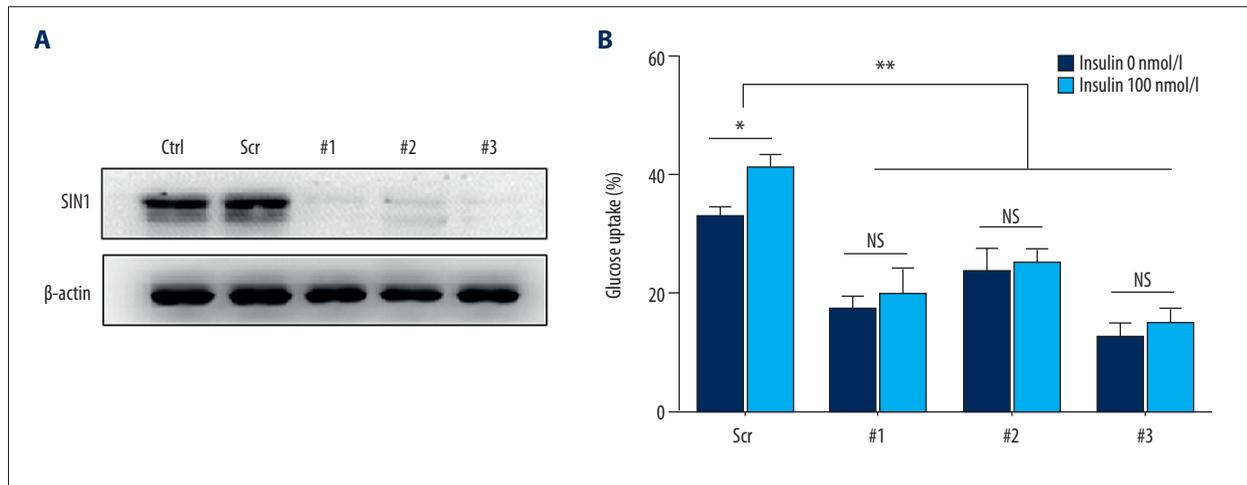
Figure 1. SIN1 downregulation impaired glucose uptake in HepG2 cells. (**A**) HepG2 cells were transfected by three different sequences of siRNAs, and SIN1 expression was measured by western blotting. (**B**) After transfer by three siRNAs of SIN1 #1, #2, #3, HepG2 cells were cultured for 24 hours with or without 100 nmol/L insulin respectively and the glucose uptake ratio was compared by Glucose Oxidase Method Kit.
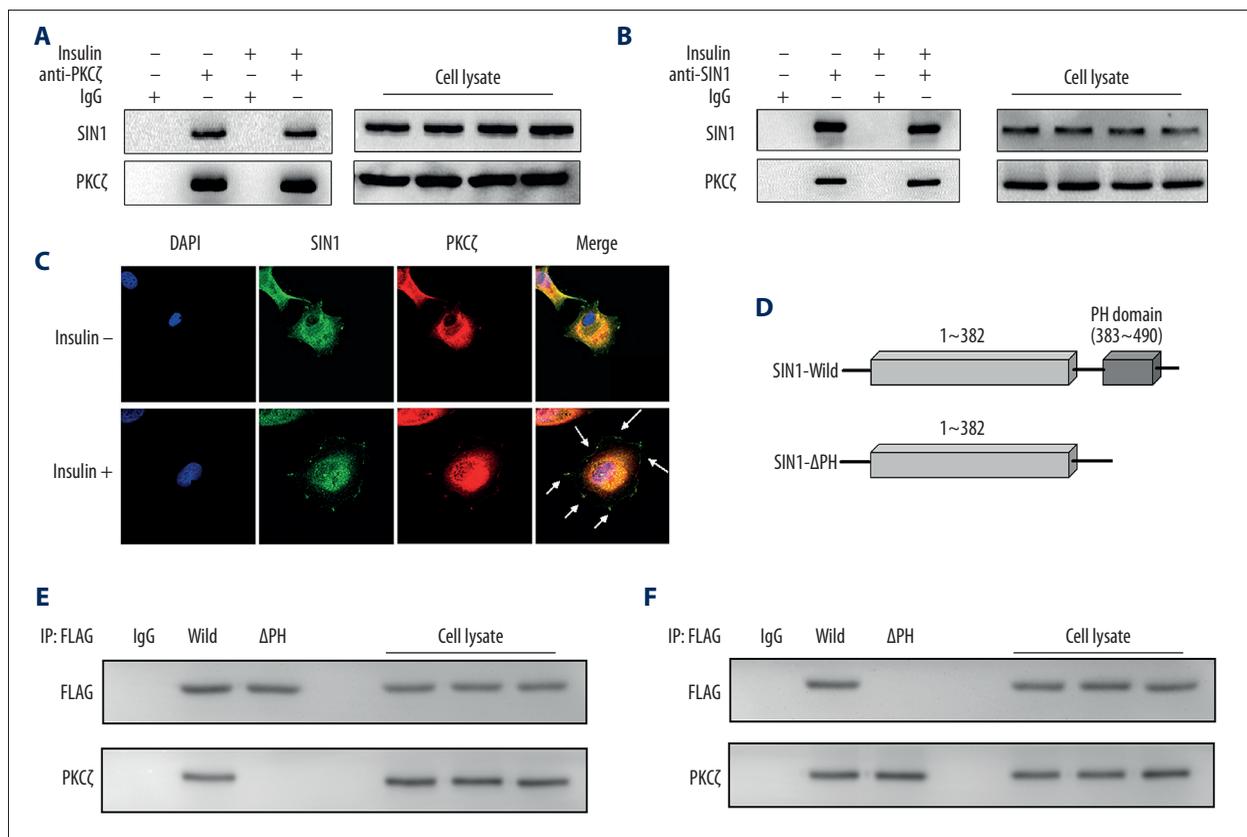
Figure 2. SIN1 co-localization with PKC ζ via its PH domain. (**A**) After stimulation with 100 nmol/L insulin for 10 minutes and co-immunoprecipitation of PKC ζ in cell lysis buffer, the expression of SIN1 was measured by western blotting. Nonimmune IgG was used as a control. (**B**) Using the same method, co-immunoprecipitation of SIN1 in lysis buffer, the expression of PKC ζ was measured by western blotting. (**C**) The co-localization relationship of SIN1 with PKC ζ in cytosol and on membranes was tested by confocal microscopy. (**D**) Sketch map of FLAG tag fused SIN1-Wild and SIN1-ΔPH proteins. (**E**) Co-IP using FLAG antibody, the expression of PKC ζ was tested by western blotting. (**F**) Co-IP using PKC ζ antibody, the expression of FLAG was tested by western blotting.
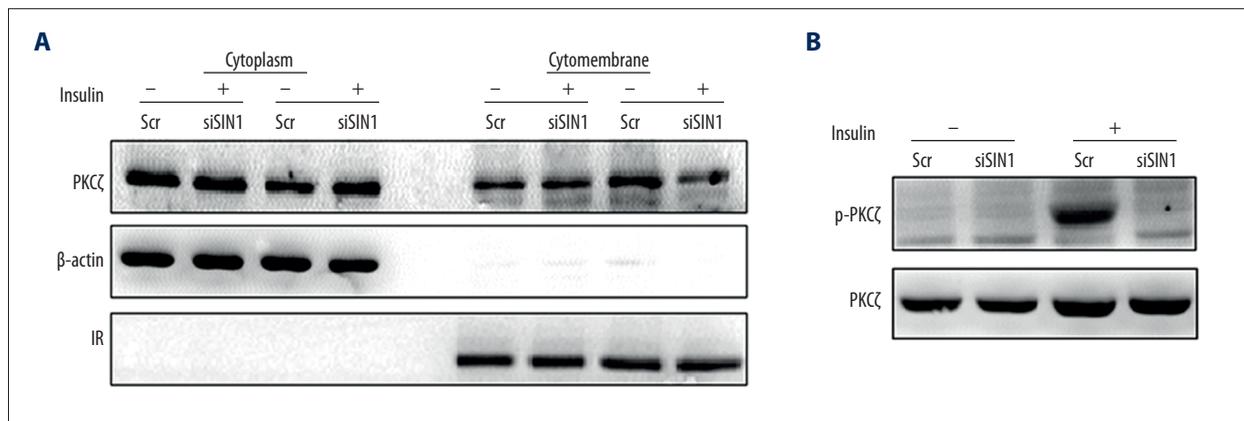
Figure 3. Downregulating SIN1 reduced PKC ζ translocation and phosphorylation. (**A**) Western blotting analysis of PKC ζ translocation from cytoplasm to the cytomembrane on stimulation with insulin for 10 minutes in both Scr and siSIN1 HepG2 cells. (**B**) Western blotting analysis of PKC ζ phosphorylation at Thr410 induced by insulin after SIN1 knockdown.

was constructed with a FLAG tag fused in the C-terminal (Figure 2D). When co-IP with FLAG antibody, PKC ζ only coprecipitated with wild SIN1 (Figure 2E). With co-IP with PKC ζ antibody, only wild SIN1 co-precipitated with PKC ζ (Figure 2F). The results indicated that PKC ζ only bound wild SIN1, but not ΔPH SIN1. PH domain deletion disrupted the interaction between SIN1 and PKC ζ, which suggested SIN1 bound PKC ζ to form a complex via its PH domain.

Downregulating SIN1 reduced PKC ζ translocation and phosphorylation

The membrane translocation of PKC ζ was analyzed in knockdown cells. Knockdown of SIN1 inhibited insulin-induced translocation from cytosol to membrane of PKC ζ. The quantity of PKC ζ in the membrane was significantly reduced under the stimulus of insulin (Figure 3A). Furthermore, insulin induced phosphorylation of PKC ζ at Thr410 was severely inhibited in siSIN1 cells (Figure 3B). These results indicated that SIN1 was required for full activation of PKC ζ.

Microtubule involved in PKC ζ activation

Most signaling proteins containing PH domain could be translocated to membrane responding to stimulus and activated on the microtubule. To detect whether microtubules were involved in PKC ζ activation, co-IP was carried out, as shown in Figure 4A: β-tubulin and PKC ζ coprecipitated with each other in resting condition *in vitro*, while the association was dim with downregulation of SIN1. These data suggested SIN1 may participate in PKC ζ activation. To detect the relationship between PKC ζ and tubulin in the insulin signal pathway, co-IP using PKC ζ antibody at different points in time after insulin stimulus was carried out. As shown in Figure 4B, the interaction between β-tubulin and PKC ζ became weaken from five minutes to 20 minutes and recovered at 30 minutes after

stimulated by insulin, while the combination of SIN1 and PKC ζ was independent of insulin stimulus. PTX was the microtubule stabilizer drug that we used to inhibit microtubule dynamics and NDZ was used to depolymerize the microtubule. When treated with 10 nM PTX and 10 µM NDZ for four hours, respectively, insulin-induced PKC ζ membrane translocation was significantly decreased (Figure 4C). Insulin-induced PKC ζ phosphorylation at Thr410 was slightly inhibited, and the phosphorylation of its downstream effect factor, cofilin, was also affected (Figure 4D). These results indicated that microtubules may affect the activation of PKC ζ. Confocal microscopy analysis showed that PKC ζ may be divorced from microtubules and transferred to the plasma membrane on stimulation with 100 nmol/L insulin for 10 minutes (Figure 4E).

Discussion

Previous works showed that PKC ζ played an important role in insulin-stimulated glucose transport through the PI3K/PKC ζ pathway [5,6], and PKC ζ may be activated directly by PI3K in the cytomembrane [10]. However, valid evidence is absent and the mechanism of PKC ζ membrane translocation remains unclear. In this study, SIN1, a unique subunit of mTORC2 which could promote cells growth and metastasis [25], was found to affect insulin-induced glucose uptake. Among the results of co-IP with SIN1, PKC ζ was identified. Besides, SIN1 could co-precipitate with PKC ζ antibody independent of insulin. The co-localization of SIN1 and PKC ζ in cytoplasm without insulin stimulus or cytomembrane with insulin stimulus, further confirmed the interaction between SIN1 and PKC ζ. In another co-IP result, PKC ζ could bind wild SIN1, but not SIN1 deleting PH domain, correlating with the results of C-terminal truncated SIN1β [26], which suggested SIN1 interacted with PKC ζ via its PH domain. In further studies, knockdown of SIN1 inhibited PKC ζ translocation and full activation. Considering

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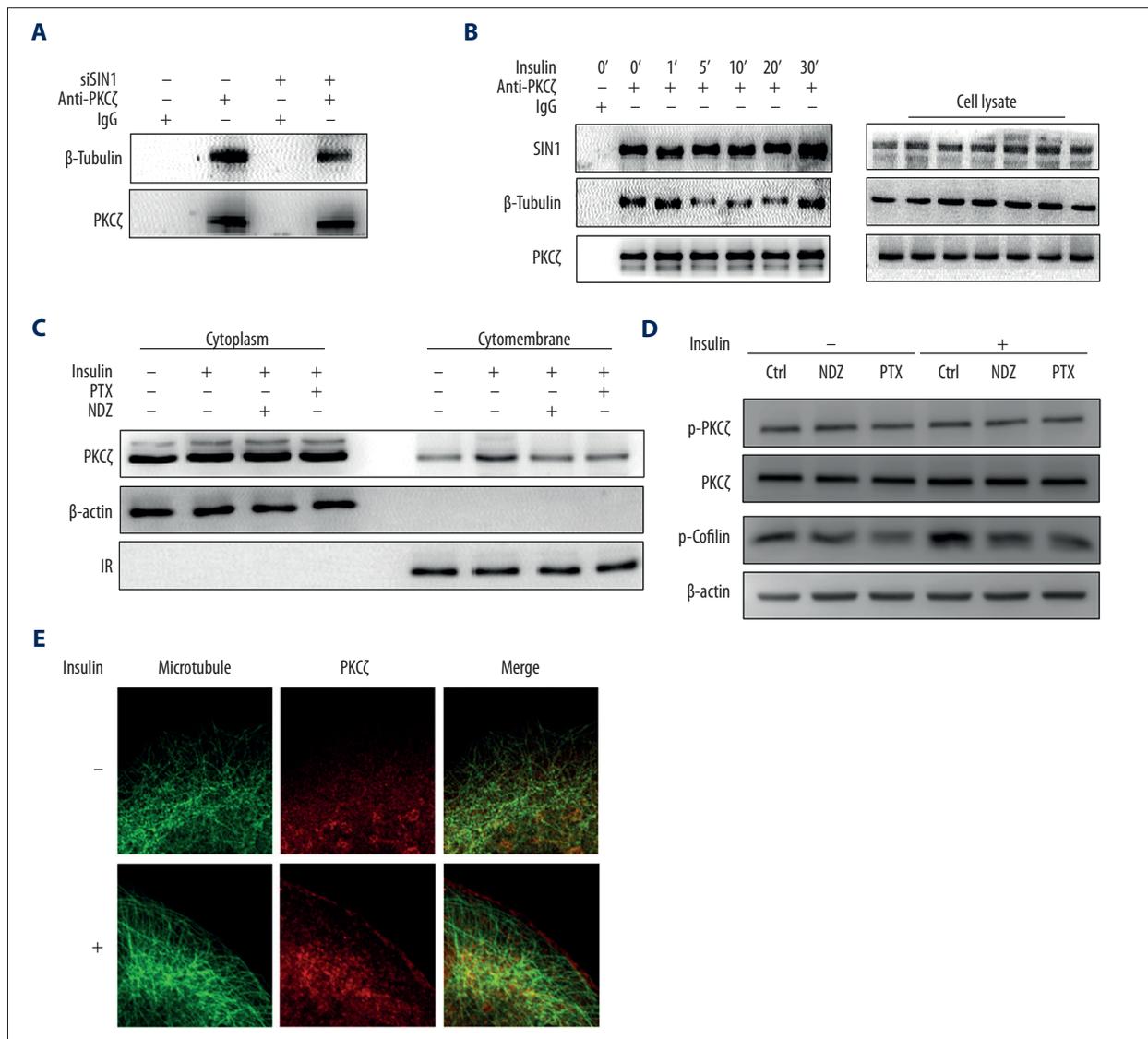
Figure 4. Microtubule involved in PKC ζ activation. (**A**) In resting condition, co-immunoprecipitation of PKC ζ in Scr and siSIN1 cells lysis buffer, and the expression of β-tubulin was measured by western blotting. Nonimmune IgG was used as a control. (**B**) SIN1 and β-tubulin co-IP with PKC ζ antibody at different intervals stimulated by 100 nmol/L insulin. (**C**) After being treated with 10 nM PTX and 10 µM NDZ for four hours, membrane translocation of PKC ζ induced by insulin was tested. (**D**) After being treated by 10 nM PTX and 10 µM NDZ, phosphorylation of PKC ζ and downstream cofilin induced by insulin was measured. (**E**) The relationship of microtubules with PKC ζ was tested by confocal microscopy before and after 100 nmol/L insulin stimulation.

SIN1 contains a PH domain, which is used to translocate signal effectors to PI3K generally [19], PKC ζ probably realized its membrane translocation dealing with insulin stimulus through binding SIN1 and activated by PI3K directly.

The microtubule cytoskeleton is a highly dynamic, filamentous network underpinning cellular structure and function [27,28]. α- and β-tubulin polymerize into dynamic microtubules and provide platforms for intracellular transport. The microtubule has been proven to be involved in a variety of signaling pathway and

many proteins are activated by second messengers on microtubules, including PI3K associated downstream effectors [29]. When HepG2 cells were treated with 100 nmol/L insulin for 0, 1, 5, 10, 20, and 30 minutes, the quantity of SIN1 that coprecipitated with PKC ζ did not change, indicating the interaction between SIN1 and PKC ζ was independent of insulin stimulus. However, the quantity of β-tubulin that coprecipitated with PKC ζ severely declined from five minutes to 20 minutes, suggesting unactivated PKC ζ had interaction with microtubules and activated PKC ζ may be detached from microtubules. PTX is

usually used to inhibit microtubular dynamics and then affect intracellular transport [30], while NDZ is used to depolymerize microtubules [31]. In our study, when treated with PTX or NDZ, the membrane translocation and phosphorylation of PKC ζ were both impaired. The phosphorylation of cofilin, serving as downstream effector of PKC ζ, was also decreased, which further confirmed the inactivation of PKC ζ induced by PTX and NDZ. These results suggested that microtubules probably served as the platform for the activation of PKC ζ by PI3K.

Conclusions

Our results revealed a novel mechanism of PKC ζ membrane translocation induced by insulin through binding SIN1 in HepG2

cells and its activation by PI3K on the platform of microtubules. In addition, the role of SIN1 in glycometabolism of human hepatocytes was further illustrated. SIN1 may be used as a novel biomarker or target to develop new metastasis therapy.

Conflicts of interest

None.

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