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Insulin Induces C2C12 Cell Proliferation and Apoptosis Through Regulation of Cyclin D1 and BAD Expression

Cheng-Juan Xiong,[1] Ping-Fa Li,[2] Yang-Liu Song,[1] Li-Xiang Xue,[3] Zhu-Qing Jia,[3] Chun-Xia Yao,[1] Qing-Xia Wei,[1] Shu-Feng Zhang,[4] Shan-Feng Zhang,[1] Yan-Yan Zhang,[5] Ji-Min Zhao,[1] Tian-Qi Wang,[1] Mao-Feng Guo,[1] and Ming-Xi Zang[1*]

[1]*Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Zhengzhou University, Zhengzhou City, Henan, 450001, China*

[2]*Department of Laboratory Medicine, Xinxiang Medical University, Xinxiang City, Henan, 453003, China*

[3]*Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Peking University, Beijing, 100191, China*

[4]*The People's Hospital of Henan Province, Zhengzhou University, Zhengzhou City, Henan, 450001, China*

[5]*School of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou City, Henan, 450001, China*

ABSTRACT

Insulin is a secreted peptide hormone identified in human pancreas to promote glucose utilization. Insulin has been observed to induce cell proliferation and myogenesis in C2C12 cells. The precise mechanisms underlying the proliferation of C2C12 cells induced by insulin remain unclear. In this study, we observed for the first time that 10 nM insulin treatment promotes C2C12 cell proliferation. Additionally, 50 and 100 nM insulin treatment induces C2C12 cell apoptosis. By utilizing real-time PCR and Western blotting analysis, we found that the mRNA levels of cyclinD1 and BAD are induced upon 10 and 50 nM/100 nM insulin treatment, respectively. The similar results were observed in C2C12 cells expressing GATA-6 or PPARα. Our results identify for the first time the downstream targets of insulin, cyclin D1, and BAD, elucidate a new molecular mechanism of insulin in promoting cell proliferation and apoptosis. J. Cell. Biochem. 114: 2708–2717, 2013.

KEY WORDS: INSULIN; CELL GROWTH; CELL APOPTOSIS; CYCLIN D1; BAD

Insulin is a secreted peptide hormone produced in the pancreas. Like other peptide hormones, insulin exerts its potent effects via signal transduction pathway, which is initiated when insulin binds to the insulin receptor, and subsequently starts a series of protein activation cascades, including Shc, phosphatidylinositol 3′-kinase (PI3K), and the mitogenactivated protein kinase (MAPK) [Zick et al., 1983a,b; Kahn and White, 1988; White and Kahn, 1994; Dandekar et al., 1998]. During the past decade accumulated data documented the critical roles of insulin in controlling energy metabolism. It is well-established that insulin induces glucose transporter Glut-4 translocation, glucose uptake, glycogen synthesis, and fatty acid synthesis [Tsao et al., 1996; Ryder et al., 2003]. Recently, insulin draws more and more attention in the aspect of cell proliferation and apoptosis. For example, Cirri et al. [2005] demonstrated that insulin inhibits platelet-derived growth factor-induced NIH3T3 and C2C12 cell proliferation; Gezginci-Oktayoglu et al. [2012] found that nerve growth factor withdrawal causes apoptosis by decreasing insulin secretion from

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*Correspondence to: Ming-Xi Zang, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Zhengzhou University, Ke Xue Da Dao 100, Zheng Zhou 450001, China. E-mail: mzang@zzu.edu.cn

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beta cells of hyperglycemic rats. The dual roles of insulin in cell proliferation and apoptosis urge us to further explore the underlying mechanisms.

GATA-6 is a zinc finger transcription factor that plays an important role in the formation of the cardiac outflow tract and aortic arch [Tsao et al., 1996; Ryder et al., 2003]. GATA-6 is essential for cardiac myocyte differentiation and maintains differentiated phenotype in vascular smooth muscle cells because it regulates the onset of cardiac–and smooth muscle–specific gene expression [Zick et al., 1983a; White and Kahn, 1994; Zang et al., 2004; Kim et al., 2007]. Moreover, our previous studies demonstrate that GATA-6 not only regulates glucose transporter Glut4 gene expression, but also stimulates both basal and insulin-stimulated glucose consumption [Yao et al., 2012].

Like GATA-6, peroxisome proliferator-activated receptor α (PPARα) serves as a critical regulator of lipid metabolism including fatty acid transport and β-oxidation [Burri et al., 2010]. In skeletal muscle over-expressing PPARα, lipid metabolisms were upregulated [Asai et al., 2006]. In addition to known regulator of lipid metabolism by PPARα, our previous studies also demonstrated the roles of nuclear receptor PPARα as a regulator of glucose metabolism. We found that PPARα not only induces Glut4 gene expression, but also interacts with GATA-6 to cooperatively to activate Glut4 expression. Consistent with these findings, PPARα stimulated both basal and insulin-stimulated glucose consumption. Moreover, C2C12 cells overexpressing GATA-6 and PPARα shows a synergistic enhancement of glucose consumption [Yao et al., 2012]. Given that both GATA-6 and PPARα are involved in the roles of insulin in glucose consumption, it would be interesting to determine whether GATA-6 and PPARα are involved in the roles of insulin in cell proliferation and apoptosis.

Apoptosis is regulated by the BCL-2 (B-cell lymphoma-2) family protein and BAD (BCL-2-antagonist of cell death) protein is one member of BCL-2 family and induces apoptosis. Upon proapoptotic signals stimulation, the transcription level of BAD was upregulated to gain its full proapoptotic function [Sermeus et al., 2012]. Moreover, apoptosis induction may be result of the anti-proliferative effect, which subsequently changes cell cycle distribution regulated by the cyclin proteins through activating cyclin-dependent kinases (CDKs) [Spizzo et al., 2010]. Cyclin D1 is a D-type cyclin and associates with CDK4 and CDK6 to stimulate the cell to enter the cell cycle [Tamamori-Adachi et al., 2008a]. Recent studies suggest that cyclin D1 proteins are involved in cardiac proliferation [Nemer and Horb, 2007; Tamamori-Adachi et al., 2008b; Nakajima et al., 2011], but relatively little is known about the expression of cyclin D1 in C2C12 cells subjected to insulin stimulation.

Here, we demonstrate that insulin promotes C2C12 cell proliferation in a dose-dependent manner, 10 nM insulin induces cell growth, whereas 50 and 100 nM insulin induces C2C12 cell apoptosis and arrests the cell cycle. By utilizing real-time PCR and Western blotting analysis, we found that the mRNA levels of cyclin D1 and BAD was upregulated upon 10 and 50 nM/100 nM insulin treatment, respectively. Our results indicate that insulin regulates cell proliferation and apoptosis through activation of its downstream targets cyclin D1 and BAD.

MATERIALS AND METHODS

RNAI PLASMIDS CONSTRUCT

The RNAi cyclin D1#1, #2 and RNAi BAD#1, #2 constructs were designed according to the pSilencer neo™ Instruction Manual (Ambion, Foster, CA) as described previously [Zang et al., 2004]. The sequences of the primers are listed in Table I.

CELL CULTURE, TRANSFECTION, AND DIFFERENTIATION

C2C12 (ATCC) cells were grown in Dulbecco′s modified Eagle′s medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS). Transfections were carried out 24 h after plating cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer′s instructions. Briefly, C2C12 cells, or cells stably expressing GATA-6 or PPARα were transiently transfected with the human 1,000 bp cyclin D1 promoter reporter construct or 2,569 bp (−3,599 to −1,031 bp) murine BAD promoter reporter construct. Twenty-four hours post-transfection, the cells were treated with 10, 50, and 100 nM insulin for 24 h. Next, cells were harvested, and luciferase activity was measured with a Berthold LB960 luminometer. The amount of reporter was maintained at 1 μg per well of a 12-well plate, and the amount of DNA was kept constant using the empty expression vector.

To construct cells stably expressing GATA-6 and PPARα, C2C12 cells were transfected with the empty pcDNA3.1(+) vector (G418-resistant), pcDNA3-GATA-6 or pcDNA3-PPARα using Lipofectamine (Invitrogen). All cells were transfected for 24 h and treated with 400 μg/ml G418 (Amersham Pharmacia Biotech). G418-resistant colonies were selected after 2 weeks [Kim et al., 2007].

TABLE I. Primer Sequences

	Forward primer	Reverse primer
Cyclin D1	ATGAGAACAAGCAGACCATCCGCA	GCTTGACTCCAGAAGGGCTTCAAT
BAD	GCTTAGCCCTTTTCGAGGAC	GATCCCACCAGGACTGGAT
Ribosomal protein S16	TCTGGGCAAGGAGAGATTTG	CCGCCAAACTTCTTGGATTC
RNAi cyclin D1 #1	GATCCGTTGTGCATCTACACTGACTTCAAGAG AGTCAGTGTAGATGCACAACTTTTTTGGAAA	AGCTTTTCCAAAAAAGTTGTGCATCTACACTGA CTCTCTTGAAGTCAGTGTAGATGCACAA
RNAi cyclin D1 #2	GATCCACAGATTGAAGCCCT TCTGTTCAAGAGAC AGAAGGGC TTCAATCTGT TTTTTTGGAAA	AGCTTTTCCAAAAAAAACAGAT TGAAGCCCTTCT GTCTCTTGACAGAAGGGCTTCAATCTGTG
RNAi Bad #1	GATCCAGACGCTAGTGCTACAGATTTCAAGAGAA TCTGTAGCACTAGCGTCTTTTTTTGGAAA	AGCTTTTCCAAAAAAAGACGCTAGTGCTACAGAT TCTCTTGAAATC TGTAGCACTAGCGTCTG
RNAi Bad #2	GATCCGTGAGTTTGAGGGTTCCTTCTTCAAGAGAG AAGGAACCCTCAAACTCATTTTTTGGAAA	AGCTTTTCCAAAAAATGAGTTTGAGGGTTCCTTCT CTCTTGAAGAA GGAACCCTCAAACTCA CG

QUANTITATIVE REAL-TIME PCR

RNA was extracted from C2C12 cells, or cells stably expressing GATA-6 or PPARα treated with 10, 50, and 100 nM insulin for 24 h using the TRIzol method (Invitrogen). First strand cDNAs were synthesized and used as a template in subsequent real-time PCR reactions as described previously [Yao et al., 2012]. A comparative quantification method was used, and the levels of mRNA were normalized to those of the ribosomal protein S16. The sequences of the primers are listed in Table I.

WESTERN BLOT ANALYSIS

Cell lysates were prepared from C2C12 cells, or cells stably expressing GATA-6, or PPARα treated with 10, 50, and 100 nM insulin for 24 h as previously described [Yao et al., 2012]. Western blotting was performed using cell lysates according to standard protocols as previously described [Yao et al., 2012]. Briefly, proteins were separated by SDS–PAGE, transferred to PVDF membranes and subjected to immunoblotting using antibodies specific for cyclin D1 (Santa Cruz, CA), BAD (Santa Cruz), or tubulin (Santa Cruz) and visualized using the standard ECL protocol (Pierce, Rockford, IL).

CELL PROLIFERATION ASSAY

C2C12 cells, or cells stably expressing GATA-6 or PPARα were seeded at 3,000 cells per well in a 96-well plate. After 24 h, the cells reached approximately 70% confluence and were treated with 10, 50, and 100 nM insulin for 24 h, and an MTT assay was performed to monitor the growth rate of these cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma). Briefly, 20 μl of a 5 mg/ml MTT solution was added to each well and incubated for 4 h at 37°C until a purple precipitate was visible. The MTT medium was then removed, and 150 μl of DMSO was added to dissolve the formazan. The absorbance was read at 490 nm using a Bio-Rad 550 Microplate Reader. All experiments were plated in sextuplicate and were performed three times.

FLOW CYTOMETRIC ANALYSIS

Flow cytometry was performed as previously described [Kim et al., 2007]. In brief, C2C12 cells, and cells stably expressing GATA-6 or PPARα were seeded at 5×10^5 cells per well in a six-well plate. After 24 h, the cells were treated with 10, 50, and 100 nM insulin for 24 h, and the cells were harvested and fixed with 70% ice-cold ethanol overnight at −20°C. After centrifugation and washing with ice-cold PBS, the cells were stained with propidium iodide (PI, Sigma) solution for 30 min at room temperature in the dark. Flow cytometric analysis was performed using a BD FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA), and cell cycle and apoptosis were analyzed using Cell-Quest software (BD Biosciences). The experiments were performed three times in duplicate.

DAPI STAINING AND ANNEXIN V-FITC/PI APOPTOSIS ASSAY

C2C12 cells, and cells stably expressing GATA-6 or PPARα were allowed to adhere to glass coverslips (22 mm × 22 mm) in six-well plates. Twenty-four hours later, the cells were treated with 10, 50, and 100 nM insulin for 24 h, and the cells on the glass coverslips were rinsed with PBS and fixed with 95% ethanol for 30 min at room temperature. The cells were DNA stained with 0.5 μg/ml DAPI (Sigma) solution for 15 min at room temperature in the dark. The coverslips were mounted and imaged with a Nikon Eclipse E800 fluorescent microscope. Apoptosis was quantified with Annexin V-FITC/PI apoptosis detection kit (KeyGen BioTECH) as previously described [Yao et al., 2013].

STATISTICS

The data are reported as the mean ± SEM. Analysis of variance (ANOVA) was used to compare differences between the three different cell types and different concentrations of insulin. In all cases, differences were considered to be statistically significant when $P < 0.05$.

RESULTS

INSULIN INDUCES C2C12 CELLS PROLIFERATION AND APOPTOSIS IN A DOSE-DEPENDENT MANNER

To elucidate the specific role of insulin in cell growth, C2C12 cells were treated with 10, 50, and 100 nM insulin for 24 h and subsequently plated in 96-well plates to measure cell viability by the MTT assay (Fig. 1A). Interestingly, 10 nM insulin treatment showed a 76% increase in cell viability, whereas 50 and 100 nM insulin treatment showed a 21% and 32% reduction in cell viability, respectively, statistical analysis indicate significant differences between untreated and 10 nM insulin-treated cells (Fig. 1A). These data suggested that insulin has both proliferative and anti-proliferative effect on C2C12 cells in a dose-dependent manner. To further determine whether the anti-proliferative effect of 50 nM/100 nM insulin is specific for C2C12 cells, a mouse myoblast cell line which can differentiate into contractile myotubes, or it is generalizable to a target cell type that has a more robust proliferative response such as NIH-3T3 fibroblasts, we did the MTT assays in NIH 3T3 cells, as shown in Figure 1B, similar results were observed. Exposure to 10 nM insulin resulted in a 73% increase in cell viability, whereas exposure to 50 and 100 nM insulin resulted in a 23% and 36% reduction in cell viability, respectively (Fig. 1B). Moreover, these results showed significant differences between untreated and 10, 50, or 100 nM insulin-treated cells (Fig. 1B), suggesting that anti-proliferative effect of 50 nM/100 nM insulin is generalizable to both differentiated and non-differentiated cell type.

To determine whether the anti-proliferative effects of 50 and 100 nM insulin is due to the cell cycle arrest, we performed FACS analysis following propidium iodide (PI) staining of C2C12 cells treated with 10, 50, and 100 nM insulin for 24 h. In comparison to control cells, the G0/G1 population decreased by 24% and the G2/M population increased by 37% in C2C12 cells treated with 10 nM insulin (Fig. 1C), suggesting that 10 nM insulin induces cell cycle. In contrast to 10 nM insulin treatment, 50 and 100 nM insulin treatment showed a 66% and 71% increase in S population, and a 7% and 12% decrease in G2/M population (Fig. 1C), suggesting that 50 and 100 nM insulin induce S-phase arrest.

To determine whether the anti-proliferative effects of 50 and 100 nM insulin results from apoptosis, we examined the effect of insulin on apoptosis in C2C12 cells treated with 10, 50, and 100 nM

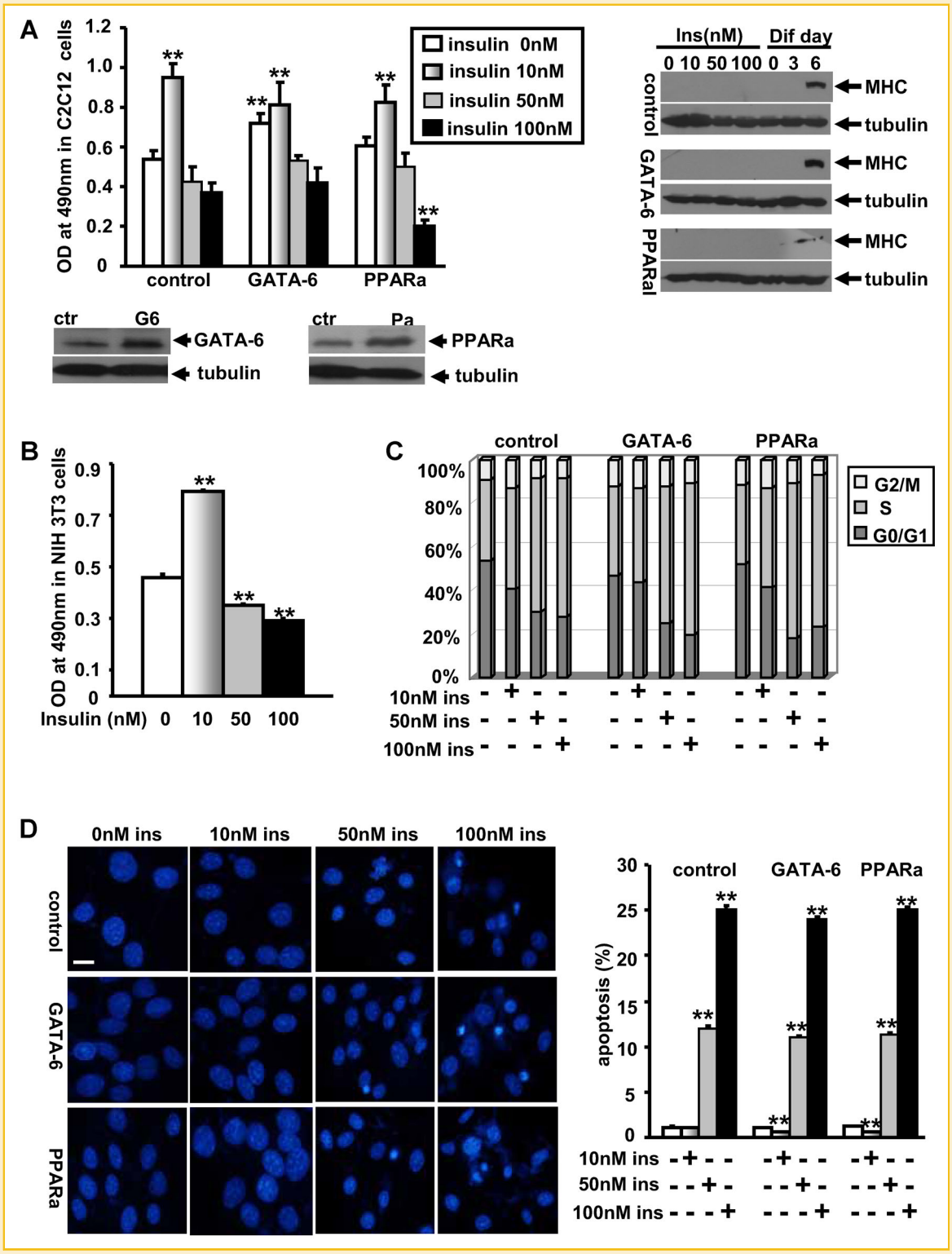

Fig. 1. The effect of Insulin on cell proliferation. A,B: C2C12 cells, and cells stably expressing GATA-6 or PPARα (A) or NIH3T3 cells (B) were seeded in 96-well plates and treated with 10, 50, and 100 nM insulin for 24 h. Cell viability was determined by MTT assay as described in the Materials and Methods Section. GATA-6 and PPARα expression levels were analyzed by Western blot analysis (lower). Ctr, control; G6, GATA-6; Pα, PPARα. The expression of sarcomeric MHC, a muscle-specific gene was analyzed by immunoblot analysis in C2C12 cells treated as described in (A) and differentiated for 3 days and 6 days (right). Ins, insulin; dif, differentiation. The data shown are the mean ± SEM of three independent experiments. $^{*}P < 0.05$ versus control, and $^{**}P < 0.01$ versus control. C: The effect of insulin on cell cycle progression. C2C12 cells, GATA-6- or PPARα-expressing C2C12 cells were treated with 10, 50, and 100 nM insulin for 24 h and subjected to FACS analysis. Ins, insulin. D: The effect of insulin on apoptosis. C2C12 cells, and cells stably expressing GATA-6 or PPARα C2C12 cells were treated with 10, 50, and 100 nM insulin for 24 h and subjected to DAPI staining (left) and Annexin V-FITC–propidium iodide (PI) double staining (right) to monitor chromatin fragmentation as an indicator of apoptosis (left) and to quantify the percentage of apoptotic cells (right), respectively. Images were taken using a 20× objective. Scale bar is 50 μm and applies to all images in (D) (left). The results are the mean ± SEM of three independent experiments. $^{**}P < 0.01$ compared to control. Ins, insulin.

insulin for 24 h by evaluating chromatin condensation. Cells treated with 50 and 100 nM insulin treatment had higher levels of condensed chromatin than the control cells, whereas 10 nM insulin treatment showed no apoptosis (Fig. 1D, left), suggesting that insulin induces C2C12 cells apoptosis in a dose-dependent manner. Consistent with these observations, FACS analysis using annexin V-FITC–propidium iodide (PI) double staining showed a 10.6- and 22-fold increase in the percentage of apoptotic C2C12 cells treated with 50 and 100 nM insulin, respectively (Fig. 1D, right), suggesting that high insulin concentrations induce apoptosis in C2C12 cells.

Taken together, these results indicated that high concentration of insulin (50 and 100 nM) inhibits C2C12 cell growth by induction of cell cycle arrest and apoptosis, and low concentration of insulin (10 nM) induces C2C12 cell growth by promoting cell cycle progression.

GATA-6- AND PPARA-INDUCED C2C12 CELL PROLIFERATION WAS FURTHER PROMOTED BY 10 NM INSULIN TREATMENT AND REVERSED BY 50 AND 100 NM INSULIN TREATMENT

Our previous studies demonstrated that GATA-6 and PPARα stimulates insulin-stimulated glucose consumption [Yao et al., 2012]. To further explore the roles of GATA-6 and PPARα in C2C12 cell growth upon insulin stimulation, the MTT assay was performed in C2C12 cells over-expressing GATA-6 or PPARα and/or subjected to insulin stimulation. As shown in Figure 1A, the expression of GATA-6 and PPARα in each stable cell line was determined by immunoblot analysis (Fig. 1A, lower). Compared with the control cells, both GATA-6 and PPARα induced cell growth, and this induction was further enhanced by 10 nM insulin treatment but reversed by 50 and 100 nM insulin treatment (Fig. 1A, left), suggesting high concentration of insulin (50 and 100 nM) reverses GATA-6- or PPARα-induced cell growth. Furthermore, compared with the control cells, significant differences were observed in C2C12 cells over-expressing GATA-6 and/or treated with 10 nM insulin, or C2C12 cells over-expressing PPARα treated with 10 or 100 nM insulin (Fig. 1A). To further determine whether C2C12 cells still are myoblasts 48 h after plating, the expression of MHC, a muscle-specific gene was analyzed by immunoblot analysis. As shown in Figure 1A (right), when compared to C2C12 cells on differentiation day 6, there are no expression of MHC in C2C12 cells, and cells stably expressing GATA-6 or PPARα and/or treated with 10, 50, and 100 nM insulin, suggesting that these cells still are myoblasts.

GATA-6 and PPARα not only induces cell growth, but also promoted cell cycle progression, and this promotion was further enhanced by 10 nM insulin treatment but reversed by 50 and 100 nM insulin treatment (Fig. 1C), suggesting high concentration of insulin (50 and 100 nM) can reverse GATA-6 or PPARα-induced cell cycle arrest. Furthermore, both GATA-6 and PPARα cannot rescue from cell apoptosis induced by 50 and 100 nM insulin (Fig. 1D), suggesting that C2C12 cell apoptosis induced high concentration of insulin does not depend on the expression of GATA-6 and PPARα.

TEN NANOMOLAR AND 50 NM/100 NM INSULIN ACTIVATES THE TRANSCRIPTION OF CYCLIN D1 AND BAD, RESPECTIVELY

To explore the molecular mechanism whereby insulin induces C2C12 cell proliferation, we performed luciferase reporter assay using a cyclin D1 promoter luciferase construct in C2C12 cells treated with 10, 50, and 100 nM insulin for 24 h. Compared with the control cells, luciferase activity of cyclin D1 promoter was increased 72% in C2C12 cells treated with 10 nM insulin, and reduced by approximately 53% and 78% in cells treated with 50 and 100 nM insulin. Moreover, these results showed significant differences between untreated and 10, 50, or 100 nM insulin-treated cells (Fig. 2A). Similar results were observed in GATA-6 or PPARα-overexpressing cells, and compared with the control cells, both GATA-6 and PPARα enhanced the promoter activity of cyclin D1 (Fig. 2A). Importantly, both GATA-6 and PPARα can only reverse the reduction of luciferase activity induced by 50 nM insulin, but not for 100 nM insulin. Moreover, compared to the control cells, significant differences were observed in C2C12 cells over-expressing GATA-6 and/or treated with 10 or 100 nM insulin, or C2C12 cells over-expressing PPARα treated with 10 or 100 nM insulin (Fig. 2A). These results demonstrate that 10 nM insulin activates cyclin D1 transcription but 50 and 100 nM insulin inhibit cyclin D1 transcription.

To explore the molecular mechanism whereby insulin induces C2C12 cell apoptosis, we performed luciferase reporter assay using a BAD promoter luciferase construct in C2C12 cells treated with 10, 50, and 100 nM insulin for 24 h. Compared with the control cells, luciferase activity of BAD promoter was reduced 33% in C2C12 cells treated with 10 nM insulin, and increased by approximately 78% and 1.1-fold in cells treated with 50 and 100 nM insulin. Moreover, these results showed significant differences between untreated and 10, 50,

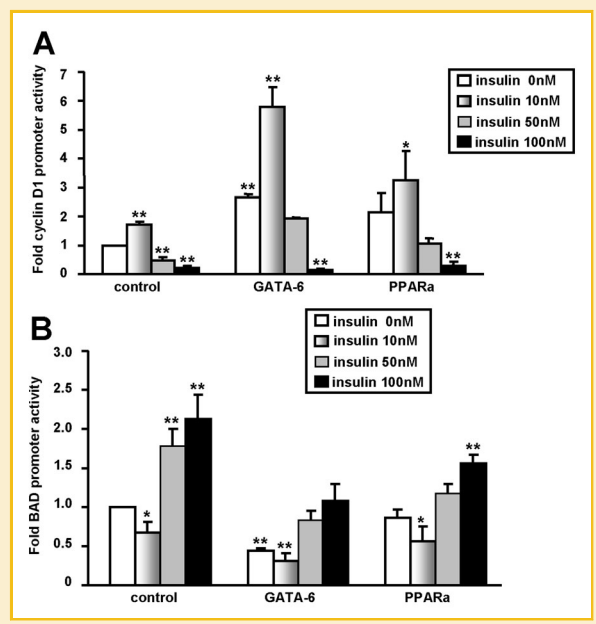

Fig. 2. The effect of Insulin on the activity of the cyclin D1 and BAD promoter. A human cyclin D1 promoter luciferase construct (−1,000 bp) (A) or A mouse BAD promoter luciferase construct (−2,568 bp) (B) was transfected in C2C12 cells, and cells stably expressing GATA-6 or PPARα, after 24 h of 10, 50, and 100 nM insulin treatment, luciferase activity was analyzed. The data shown are the mean ± SEM of three independent experiments carried out in duplicate (out of 3). $^{*}P < 0.05$ versus control.

or 100 nM insulin-treated cells (Fig. 2B). Similar results were observed in GATA-6 or PPARα-overexpressing cells, and compared to the control cells, significant differences were observed in C2C12 cells over-expressing GATA-6 and/or treated with 10 nM insulin, or C2C12 cells over-expressing PPARα treated with 10 or 100 nM insulin (Fig. 2B). Importantly, both GATA-6 and PPARα inhibits the luciferase activity induced by 50 and 100 nM insulin (Fig. 2B). These results demonstrate that 50 and 100 nM insulin activate BAD transcription but 10 nM insulin inhibits BAD transcription.

TEN NANOMOLAR AND 50 NM/100 NM INSULIN INDUCES THE EXPRESSION OF CYCLIN D1 AND BAD, RESPECTIVELY

To determine whether insulin induces the mRNA level of cyclin D1 and BAD in a dose-dependent manner, we treated C2C12 cells with 10, 50 and 100 nM insulin for 24 h, and then the mRNA levels of cyclin D1 and BAD were determined by Quantitative real-time PCR (Q-PCR) experiments. As shown in Figure 3, the cyclin D1 mRNA level was reduced by 82% in 50 nM insulin-treated cells and by 84% in 100 nM insulin-treated C2C12 cells (Fig. 3A), but was increased by 78% in 10 nM insulin-treated C2C12 cells (Fig. 3A). Similar results were observed in GATA-6-overexpressing cells, but in PPARα-overexpressing cells, 50 nM insulin treatment resulted in almost the same decreases in cyclin D1 mRNA level as 100 nM insulin treatment. Moreover, GATA-6 induces cyclin D1 mRNA level (Fig. 3A). Statistical analysis indicate there are significant differences between control cells and other treated cells except PPARα-overexpressing cells (Fig. 3A). Moreover, both GATA-6 and PPARα cannot rescue the reduction of cyclin D1 mRNA level mediated by 50 and 100 nM insulin (Fig. 3A). These results demonstrate that insulin induces the mRNA level of cyclin D1 in a dose-dependent manner.

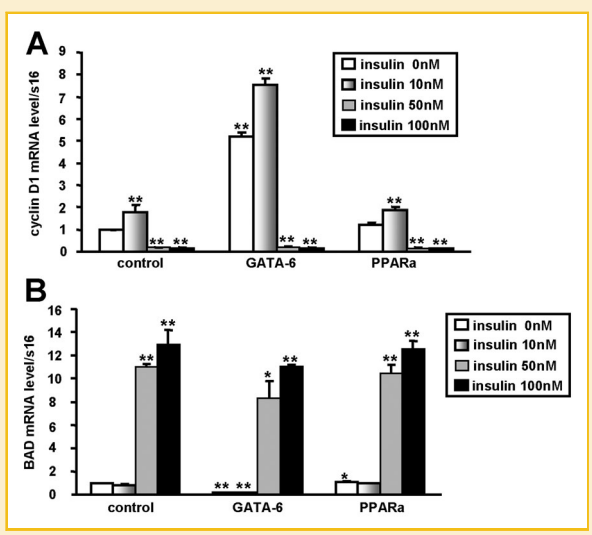

Fig. 3. The cyclin D1 and BAD mRNA levels response to insulin treatment. C2C12 cells, and cells stably expressing GATA-6 or PPARα were treated with 10, 50, and 100 nM insulin for 24 h, and expression levels of cyclin D1 (A) and BAD (B) were evaluated. The results are the mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control.

In contrast to cyclin D1, the BAD mRNA level was increased 10-fold in 50 nM insulin-treated cells and 11.9-fold in 100 nM insulin-treated C2C12 cells (Fig. 3B), but was downregulated in 10 nM insulin-treated C2C12 cells (Fig. 3B). Similar results were observed in PPARα-overexpressing cells, but in GATA-6-over-expressing cells, with or without 10 nM insulin treatment resulted in almost the same decreases in BAD mRNA level. Moreover, GATA-6 inhibits BAD mRNA level (Fig. 3B). Statistical analysis indicates there are significant differences between control cells and other treated cells except C2C12 cells or PPARα-over-expressing cells treated with 10 nM insulin (Fig. 3B). Moreover, both GATA-6 and PPARα neither rescue the reduction of BAD mRNA level mediated by 10 nM insulin, nor fully reverse the increase of BAD mRNA level mediated by 50 and 100 nM insulin (Fig. 3B). These results demonstrate that insulin induces the mRNA level of BAD in a dose-dependent manner.

Consistent with the observed effects of insulin at the mRNA levels, the protein levels of cyclin D1 were increased in cells treated with 10 nM insulin and decreased in cells treated with 50 and 100 nM insulin, similar results were observed in GATA-6 or PPARα-overexpressing cells (Fig. 4), whereas the protein levels of BAD were increased in C2C12 cells treated with 50 and 100 nM insulin, and GATA-6 or PPARα over-expressing cells treated with 50 and 100 nM insulin (Fig. 4, Table II). These data demonstrated that insulin induces the expression of cyclin D1 and BAD in a dose-dependent manner.

THE EFFECT OF CYCLIN D1 OR BAD KNOCKDOWN IN INSULIN-MEDIATED CELL GROWTH

To elucidate the specific role of cyclin D1 and BAD in insulin-mediated cell growth, cyclin D1(#2) or BAD (#1) knockdown C2C12 cells were treated with 10, 50, and 100 nM insulin for 24 h and subsequently plated in 96-well plates to measure cell viability by the MTT assay (Fig. 5A, upper). Interestingly, the proliferative and anti-proliferative effect mediated by 10 and 50 nM/100 nM insulin were reversed by the knockdown of cyclin D1 and BAD expression, respectively, and the downregulation of cyclin D1 and BAD expression were confirmed by Western blot assay (Fig. 5A, lower). These data suggested that cyclin D1 and BAD are involved in insulin-mediated cell growth.

To determine whether the anti-proliferative effects of RNAi cyclin D1 is due to the cell cycle arrest, we performed FACS analysis following propidium iodide (PI) staining of cyclin D1 (#2) or BAD (#1) knockdown C2C12 cells treated with 10, 50, and 100 nM insulin for 24 h. In comparison to control cells, the G0/G1 population increased by 16% and the G2/M population decreased by 11% in cyclin D1(#2) knockdown C2C12 cells (Fig. 5B), suggesting that cyclin D1(#2) knockdown results in cell G0/G1 arrest. Moreover, cyclin D1 knockdown not only reverses the induction of cell cycle by 10 nM insulin, but also stimulated the cell cycle arrest mediated by 50 and 100 nM insulin (Fig. 5B), suggesting that cyclin D1 plays roles in insulin-mediated cell cycle. In contrast to cyclin D1 knockdown, BAD knockdown further induces cell cycle mediated by 10 nM insulin, and reverses the cell cycle arrest mediated by 50 and 100 nM insulin (Fig. 5B), suggesting that BAD plays roles in 50 nM/100 nM insulin-mediated cell cycle.

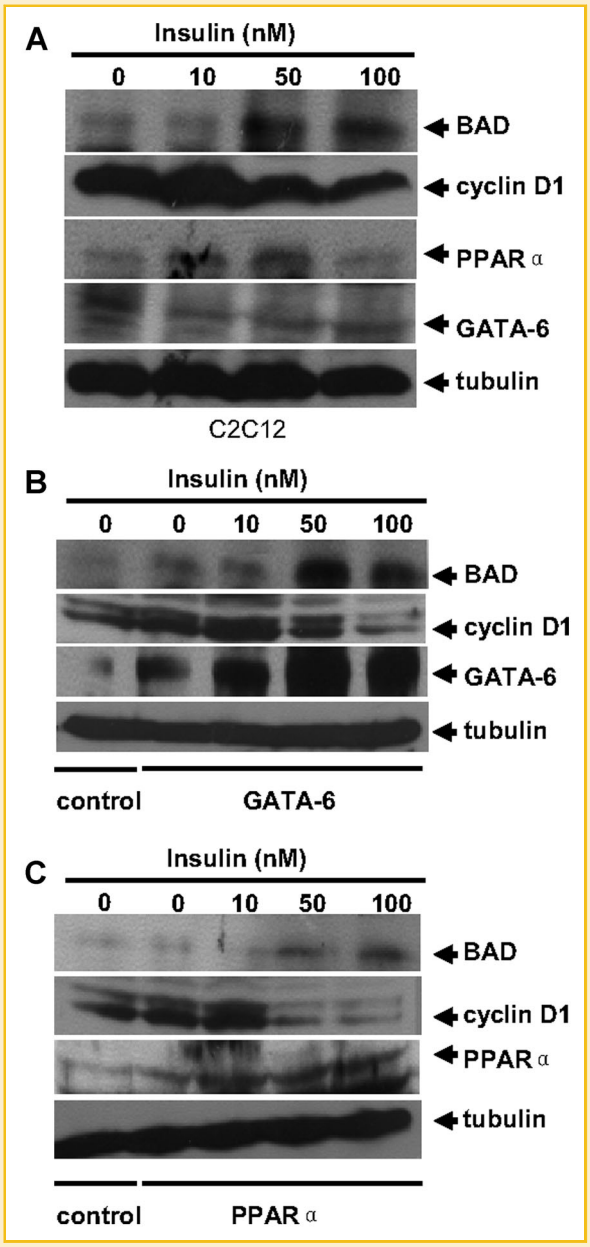

Fig. 4. Cyclin D1 and BAD protein levels response to insulin. C2C12 cells (A), and cells stably expressing GATA-6 (B) or PPARα (C) were treated with 10, 50, and 100 nM insulin for 24 h, and cyclin D1, BAD, GATA-6, PPARα, and tubulin protein levels were evaluated. The signal strength was quantified using image analysis software and was found in Table II.

TABLE II. Quantification by the FUJIFILM-Multi Gauge V3.0 Software for Figure 4

Ratio	Insulin				
Lane	0 nM	0 nM	10 nM	50 nM	100 nM
Figure 4A		C2C12 cells			
Bad		0.152248	0.140546	0.432399	0.382198
Cyclin D1		0.475075	0.544932	0.394869	0.287021
PPARa		0.146851	0.36345	0.328931	0.155743
GATA-6		0.26203	0.082846	0.114964	0.157931
Figure 4B	C2C12 cells	GATA-6 overexpressing C2C12 cells			
Bad	0.242137	0.481998	0.400527	1.172772	0.784919
Cyclin D1	1.259027	1.726341	1.927026	1.140829	0.495199
GATA-6	0.265871	1.210901	1.617925	1.580048	1.562156
Figure 4C	C2C12 cells	PPARa overexpressing C2C12 cells			
Bad	0.124453	0.146465	0.077968	0.759283	0.921947
Cyclin D1	0.522845	0.975341	1.013192	0.358923	0.211918
PPARa	0.181032	0.419012	0.87281	0.864078	0.817457

To determine whether the anti-proliferative effects of RNAi cyclin D1 results from apoptosis, we examined the effect of cyclin D1 or BAD knockdown on apoptosis in C2C12 cells treated with 10, 50, and 100 nM insulin for 24 h by evaluating chromatin condensation. Cyclin D1 knockdown cells showed no apoptosis, whereas these cells treated with 50 and 100 nM insulin had higher levels of condensed chromatin (Fig. 5C, left), suggesting that cyclin D1 knockdown does not induce C2C12 cells apoptosis. Consistent with these observations, FACS analysis using annexin V-FITC–propidium iodide (PI) double staining showed no significant increase in the percentage of apoptotic C2C12 cells after knockdown of cyclin D1 expression, compared with the control cells (Fig. 5C, right), but cyclin D1 knockdown induces apoptosis mediated by 50 and 100 nM insulin, whereas BAD knockdown reverses 50 and 100 nM insulin-mediated cell apoptosis (Fig. 5C, right), suggesting that BAD plays roles in 50 nM/100 nM insulin-mediated apoptosis in C2C12 cells.

Taken together, these results indicated that cyclin D1 and BAD are involved in insulin-mediated cell growth.

DISCUSSION

Our results show that insulin induces cell proliferation and apoptosis via regulation the expression of cyclin D1 and BAD in a dose-dependent manner. To the best of our knowledge, we are the first to describe an anti-growth and apoptosis function of insulin in C2C12 cells and to establish the downstream target of insulin, cyclin D1 and BAD, which plays important roles in cell proliferation and apoptosis.

Previous studies have shown that insulin is involved in glucose uptake and utilization in muscle and adipose tissue, which contributes to the control of whole-body blood glucose levels in humans [Tsao et al., 1996; Ryder et al., 2003]. Insulin exerts these effects through the crucial proteins, such as FOX transcription factors, estrogen receptor, and HDAC2 [Deng et al., 2008; Sun and Zhou, 2008; Gerin et al., 2009]. Recently, insulin was found to be involved in myogenesis, breast cancer, autophagy, mitochondriogenesis [Conejo et al., 2001; Pawlikowska et al., 2006; Chan et al., 2012; Yang and Yee, 2012]. Here, we show that insulin also induces C2C12 cell proliferation and apoptosis through regulation the expression of cyclin D1 and BAD in a dose-dependent manner.

Four lines of evidence indicate that insulin induces cell growth and apoptosis. First, MTT experiments indicated that 10 nM insulin treatment results in accelerate C2C12 cell growth, whereas 50 and 100 nM insulin treatment inhibit cell growth. Second, FACS analysis demonstrated that 10 nM insulin induces cell cycle, whereas 50 and 100 nM insulin induces S-phase arrest. Third, cells treated with 50 and 100 nM insulin showed higher levels of condensed chromatin,

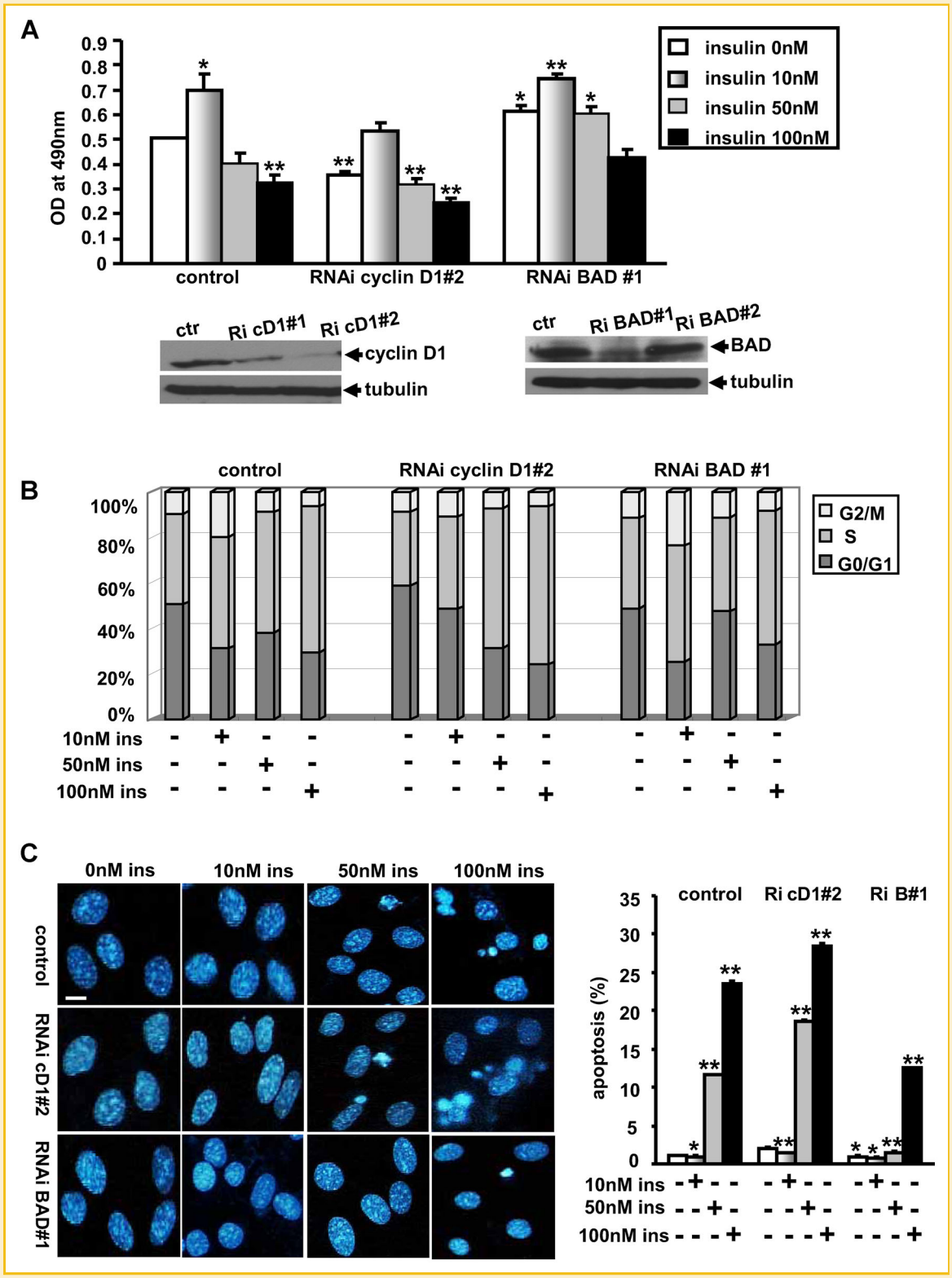

Fig. 5. The effect of cyclin D1 or BAD knockdown on insulin-mediated cell growth. A: C2C12 cells, and cyclin D1 or BAD knockdown C2C12 cells were seeded in 96-well plates and treated with 10, 50 and 100 nM insulin for 24 h. Cell viability was determined by MTT assay. cyclin D1 and BAD expression levels were analyzed by Western blot analysis (lower). Ctr, control; Ri cD1, RNAi cyclin D1; Ri BAD, RNAi BAD. (B) The effect of cyclin D1 or BAD knockdown on insulin-mediated cell cycle progression. C2C12 cells, and cyclin D1 or BAD knockdown C2C12 cells were treated with 10, 50, and 100 nM insulin for 24 h and subjected to FACS analysis. Ins, insulin. (C) The effect of cyclin D1 or BAD knockdown on insulin-mediated apoptosis. C2C12 cells, and cyclin D1 or BAD knockdown C2C12 cells were treated with 10, 50, and 100 nM insulin for 24 h and subjected to DAPI staining (left) and Annexin V-FITC–propidium iodide (PI) double staining (right) to monitor chromatin fragmentation as an indicator of apoptosis (left) and to quantify the percentage of apoptotic cells (right), respectively. Images were taken using a 20× objective. Scale bar is 50 μm and applies to all images in Figure 1D (left). The results are the mean ± SEM of three independent experiments. $^{**}P < 0.01$ compared to control. Ins, insulin; Ri cD1, RNAi cyclin D1; Ri B, RNAi BAD.

whereas 10 nM insulin treatment showed no apoptosis. Fourth, 10 nM insulin treatment results in upregulation of cell cycle activator cyclin D1 expression, whereas 50 and 100 nM insulin treatment activates the mRNA level of BAD, an activator of cell apoptosis. Given different daily insulin dosage for patients based on their age, stages of disease development, different types of diabetes such as type 1 and type 2, our results are benefit for clinicians to define optimal insulin dosages because low concentration of insulin induces cell growth and high concentration of insulin induces cell apoptosis, so our findings have clinical implications during insulin treatment.

Other studies also describe the roles of insulin in cell growth and apoptosis. For example, Cirri et al. [2005] have shown that insulin inhibits platelet-derived growth factor-induced NIH3T3 and C2C12 cell proliferation. Gezginci-Oktayoglu et al. [2012] have shown that decreasing insulin secretion causes beta cells apoptosis in hyperglycemic rats. Our studies not only are consistent with these studies describing insulin as a regulator of cell growth and apoptosis, but also further broaden our knowledge about the roles of insulin in inducing cell growth and apoptosis in a dose-dependent manner.

In addition to showing that insulin plays roles in cell growth and apoptosis, we have also found that insulin regulates the transcription and expression of cyclin D1 to effect on cell growth. The relevance of the insulin–cyclin D1 pathway in the proliferation of C2C12 cells has also been demonstrated by Yang et al. [2007], who showed that insulin-like growth factor 1 (IGF-1) treatment upregulated cyclin D1 expression and induces C2C12 cell proliferation. Here, we not only demonstrated that insulin induces cyclin D1 expression and cell growth at 10 nM concentration, but also demonstrated that insulin inhibits cyclin D1 expression and cell growth at 50 and 100 nM concentration, so the effect of the insulin–cyclin D1 pathway on the cell proliferation is a dose-dependent manner. Besides cyclin D1, we also demonstrated that insulin also regulated BAD expression in a dose-dependent manner, which results in cell apoptosis. Moreover, the enhancement or depletion of cyclin D1 mRNA level mediated by insulin is accompanied by the concurrent down- or upregulation of BAD mRNA level, respectively, which may partly account for the growth acceleration and apoptosis mediated by 10 and 50 nM/100 nM insulin, respectively. In conclusion, a regulatory cascade involving insulin and cyclin D1/BAD regulates cell growth and apoptosis.

Our previous studies demonstrated that both GATA-6 and PPARα are involved in glucose metabolism by regulation of glucose transporter Glut4 expression and stimulation insulin-stimulated glucose consumption [Yao et al., 2012]. We also found that 100 nM insulin treatment induces apoptosis in C2C12 cells, or cells expressing GATA-6 or PPARα, which urged us to determine the related mechanisms. Here, we found that GATA-6 activates and inhibits transcription of cyclin D1 and BAD, respectively, which is responsible for the acceleration of C2C12 cell proliferation mediated by GATA-6. Moreover, the expression of cyclin D1 and BAD is subject to insulin regulation. By implementing transient transfection, Q-PCR and immunoblotting analysis, we have demonstrated that the expression of cyclin D1 is downregulated by 50 and 100 nM insulin treatment. In contrast to reduced expression of cyclin D1, 50 nM insulin treatment upregulated the expression of BAD protein. Ten nanomolars of insulin treatment, on the other hand, upregulated the expression of cyclin D1. The fact that the expression of cyclin D1 and BAD can alter according to the concentration of insulin implicates a potential mechanism to switch the balance between cell growth and apoptosis.

Lastly, we found that both GATA-6 and PPARα can neither rescue the reduced expression of cyclin D1 mediated by 100 nM insulin, nor fully reverse the enhanced mRNA levels of BAD mediated by 50 and 100 nM insulin. Because insulin exerts its potent effects via signal transduction pathway, we speculate that both GATA-6 and PPARα may be not the downstream effectors and upstream regulators of insulin signaling pathway. Accordingly, further studies will be undertaken to investigate the association between insulin signaling, GATA-6 and PPARα, and cyclin D1 and BAD.

In conclusion, the effects of insulin on C2C12 cell growth and apoptosis are mediated through the regulation of cyclin D1 and BAD expression. These findings represent a novel mechanism of insulin in cell growth and apoptosis and have a new clinical implications in designing insulin therapies.

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