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Poly(ADP-Ribose) Polymerase 1 is Involved in Glucose Toxicity Through SIRT1 Modulation in HepG2 Hepatocytes

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ABSTRACT

Accelerated glucose metabolism leads to oxidative stress and DNA damage in cells; these effects are related to glucose toxicity. The precise mechanisms of glucose toxicity are still unclear. The aim of this work was to investigate the mechanism of poly(ADP-ribose) polymerase 1 (PARP1), which is a DNA repair enzyme activated by high-glucose-induced oxidative stress, and its effect on glucose toxicity in HepG2 hepatocytes. HepG2 cells were cultured under normal (5.5 mM) or high (30 mM) glucose conditions for 4 days. PJ34, which is an inhibitor of PARP1, was used to determine the downstream effects of PARP1 activation. PARP1 activity in 30 mM-glucose-treated cells was more than that in 5.5 mM-glucose-treated cells, and the activity correlated with the increase in ROS generation and DNA damage. PJ34 suppressed PARP1 activation and prevented the high-glucose-induced suppression of SIRT1 and AMP-activated protein kinase (AMPK) activity, which was similar to its effect on the restoration of intracellular nicotinamide adenine dinucleotide (NAD) content. Further, the phosphorylation of insulin receptor was attenuated in response to insulin stimulation under high glucose conditions, and PJ34 could reverse this effect. The results of transfection of HepG2 cells with PARP1 small interfering RNA were similar to those obtained by treatment of the cells with PARP1 inhibitor PJ34. These data suggest that high-glucose-induced PARP1 activation might play a role in glucose toxicity by down-regulating SIRT1 and AMPK activity through NAD depletion and resulting in insulin insensitivity. J. Cell. Biochem. 112: 299–306, 2011.

KEY WORDS: HYPERGLYCEMIA; PARP1; NAD; SIRT1; AMPK; INSULIN SENSITIVITY

The deleterious effects of chronic hyperglycemia play an important role in the pathophysiology of diabetes by causing glucose toxicity. Hyperglycemia can result in intracellular oxidative stress and trigger a series of cellular responses [Brownlee, 2001]. High glucose levels induced negative effects related to not only direct damage of biological macromolecules by reactive oxygen species (ROS), including damage to lipids, proteins, and DNA, etc., [Rösen et al., 2001] but also to cellular regulation of stress caused by ROS [Evans et al., 2003; Ogihara et al., 2004]. These changes are closely related to cell dysfunction.

Generally, high ROS levels can damage DNA structure and induce errors in transcription, translation, and cell function. The association between DNA structure damage and pathophysiological effects in diabetes has been established [Rolo and Palmeira, 2006; Xie et al., 2008]. Further, intracellular DNA damage initiates a series of self-regulated events related to DNA repair, thereby maintaining normal function and cell survival. However, recent studies have proved that DNA repair is correlated with pathophysiological effects in diabetes, and the main protein involved in this process is the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) [Szabó et al., 2002].

PARP1 is a DNA damage sensor and a signaling molecule that maintains chromatin structure and DNA stability [de Murcia et al., 1997; Shall and de Murcia, 2000]. PARP1 is activated in response to localized DNA strand breaks and utilizes nicotinamide adenine dinucleotide (NAD) to catalyze the poly(ADP-ribosyl)ation of acceptor proteins. Overactivation of PARP1 may lead to diabetic complications [Szabó et al., 2006; Horváth et al., 2009]. Continual

Abbreviations: AMPK, 5′ adenosine monophosphate-activated protein kinase; Akt, protien kinase B; GSK, glycogen synthase kinase; InsR, insulin receptor; NAC, *N*-acetyl-cysteine; NAD; nicotinamide adenine dinucleotide; PARP1, poly(ADP-ribose) polymerase 1; PJ34, potent water-soluble phenanthridinone-derived PARP inhibitor; ROS, reactive oxygen species; SIRT1, silent mating type information regulation 2 homolog 1.The authors have nothing to disclose.

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activation of PARP1 would lead to NAD and ATP depletion, and eventually cell death [Virág and Szabó, 2002; Pillai et al., 2005]. PARP1 activation, which is a self-regulated downstream response to DNA damage, plays a role in cellular dysfunction; however, most studies have focused on the role of PARP1 activation in cell necrosis or apoptosis [Pieper et al., 1999; Koh et al., 2005; Son et al., 2009]. The other effects of PARP1 activation on the pathophysiological process remain to be clarified.

To prove the hypothesis that PARP1 is involved in glucose toxicity and contributes to insulin sensitivity, we analyzed the ability of glucose-utilizing cells to respond to insulin stimulation after high-glucose treatment for 4 days. We evaluated the expression and activity of the NAD-dependent enzyme SIRT1 and the energy sensor protein AMP-activated protein kinase (AMPK) to identify the possible downstream pathway of PARP1 activation. Extracellular treatment with high-glucose induced ROS generation, initiated a series of intracellular responses to DNA damage, and finally impaired cell function. These responses may serve as a link between the regulation of cellular stress and cell dysfunction.

MATERIALS AND METHODS

MATERIALS

Anti-PARP1, anti-phospho-insulin receptor (InsR) β (Tyr1150/1151), anti-InsR β, anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-GSK-3β (Ser 9), anti-phospho- Akt (Ser 473), and anti-Akt antibodies were from Cell Signaling Technology (Danvers, MA). Mouse anti-poly(ADP-ribose) (PAR) monoclonal antibody was from Trevigen (Gaithersburg, MD). Rabbit anti-SIR2 polyclonal antibody was from Upstate (Temecula, CA). We used the following kits in this study: ROS assay kit (S0033; Beyotime, China), NAD^+/NADH quantification kit (k337; BioVision, Mountain View, CA), and SIRT1 deacetylase fluorometric assay kit (CY-1151; CycLex, Japan). *N*-Acetyl-cysteine (NAC), PJ34 (PARP1 inhibitor), mannitol, and other chemicals were from Sigma–Aldrich (St. Louis, MO) or as indicated in the text.

CELL CULTURE

Human hepatoma HepG2 cells obtained from Concord cell center (Peking Union Medical College) were cultured in Eagle's minimum essential medium (MEM; Invitrogen, Grand Island, NY) containing 5.5 mM glucose supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells were plated on 25 cm^2 flasks and grown in a 5% CO_2-humidified atmosphere at 37°C.

MEASUREMENT OF INTRACELLULAR ROS GENERATION

Cells were seeded in 24-well plates in MEM containing 5.5 mM glucose (control) or 30 mM glucose (high glucose) for 1, 2, and 4 days. In the osmolarity normalization groups, cells were cultured with 5.5 mM glucose and 24.5 mM mannitol for 4 days. Further, cells in the oxidation-protected and damage control groups were cultured with 2.5 mM NAC (ROS scavenger) and 30 mM glucose for 4 days or with 50 μg/ml Rosup (supplied by ROS assay kit) for 15 min, respectively. Subsequently, the cells were washed with phosphate-buffered saline (PBS) twice and treated with 5 μM dichlorofluorescin diacetate (DCFH-DA) for 30 min at 37°C in the dark. The cells were washed again, trypsinized, and resuspended in PBS. The principle of the ROS assay kit was based on the oxidation of the non-fluorescent DCFH into a fluorescent dye 2′,7′-dichlorofluorescein. The intensity of fluorescence was immediately measured by a flow cytometry instrument (BD, Franklin Lakes, NJ) at 488 nm excitation and 530 nm emission wavelengths.

COMET ASSAY

The cells were cultured in media containing 5.5 mM or 30 mM glucose for 2 and 4 days, respectively, and subsequently trypsinized for detecting DNA damage by comet assay [Olive and Banáth, 2006]. Briefly, an aliquot of 10^5 cells was suspended in 1% low-melting-point agarose and spread on microscopic slides precoated with normal agarose. The slides were placed in a lysis solution with high salt content for 1 h at 4°C, immersed in alkaline buffer for 40 min, electrophoresed at 0.6 V/cm for 25 min, and stained with 10 μg/ml propidium iodide for 20 min. The slides were observed microscopically and 100 images of each sample were randomly selected; olive tail moment was calculated using CASP comet assay software. A slide treated by the aforementioned procedure and incubated with Rosup (50 μg/ml) for 15 min served as a positive control.

WESTERN BLOTTING

Cells were treated with 5.5 mM or 30 mM glucose with or without PJ34 for 4 days and whole cell lysates were used for Western Blotting. To determine if InsR was phosphorylated, we stimulated cells with 100 nM insulin for 20 min before lysis. The samples (50 μg protein, as determined by Bio-Rad assay, Thermo, Rockford, IL) were separated by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and blocked with 5% milk in Tris-buffered saline tween. Anti-PAR (1:1,000 dilution), anti-PARP1 (1:1,000 dilution), anti-InsR β (1:1,000 dilution), anti-phospho-InsR β (p-Tyr1150/1151-InsR; 1:1,000 dilution), anti-AMPKα (1:1,000 dilution), anti-phospho-AMPKα (p-AMPK; 1:1,000 dilution), anti-SIRT1 (1:4,000 dilution), anti-phospho-GSK 3β (p-Ser9-GSK; 1:1,000 dilution), anti-phospho-Akt (p-Ser473-Akt; 1:1,000 dilution), anti-Akt (1:1,000 dilution), and anti-actin (1:20,000 dilution) antibodies were incubated overnight at 4°C. The bound antibodies were detected using horseradish peroxidase-linked secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized using enhanced chemiluminescence and autoradiography. Actin was used as a control to confirm equal loading of proteins. The phosphorylation of InsR and AMPK was normalized to the amount of InsR and AMPK proteins, respectively, in each sample.

ESTIMATION OF NAD LEVELS

Intracellular NAD content was measured using NAD^+/NADH quantification kit. The NAD plus NADH (NADt) and NADH contents of the samples were measured by a microplate reader (Wellscan MK3, Labsystems Dragon, Finland) at 450 nm. Then, NAD levels were calculated by subtracting the value of NADH from NADt.

SIRT1 ACTIVITY ASSAYS

According to the instruction manual of SIRT1 deacetylase fluorometric assay kit, we used a nuclear extract to measure the

deacetylation activity of SIRT1. The fluorophore and quencher were coupled to amino terminal and carboxyl terminal, respectively, of the acetylated substrate peptide. SIRT1 deacetylated the substrate; this substrate was cleaved by a protease, leading to the separation of the quencher from the fluorophore. The resulting fluorescence was measured at 340 nm excitation and 440 nm emission wavelengths by using the Safire2 microplate reader (Tecan, Switzerland).

SMALL INTERFERING RNA TRANSFECTION

Small interfering RNA (siRNA) targeting PARP1 mRNA was transiently transfected into HepG2 cells using Lipofectamin 2000 (Invitrogen, Frederick, MD) in accordance with the manufacturer's recommendations. HepG2 cells were cultured in a medium containing 30 mM glucose for 4 days, and were transfected with siRNA-PARP1 on day 3.

CELL VIABILITY ASSAYS

We inoculated 1×10^4 cells per well in a 96-well plate and incubated them for 4 days with either 5.5 mM or 30 mM glucose plus different concentrations of PJ34. For each condition, we analyzed three samples. Water-soluble tetrazolium salt-1 (WST-1; Beyotime), which is an alternative to MTT, was added to each well; subsequently, the plate was incubated for 2 h at 37°C. Cell viability was measured at 450 nm using a microplate reader (Wellscan MK3, Labsystems Dragon, Finland).

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (SD). Comparisons between two mean values were performed by independent-samples *t*-test. For multiple comparisons among different groups of data, the significant differences were determined by one-way analysis of variance (ANOVA) by using SPSS software for Windows 11.0. The difference between the values was considered significant when $P < 0.05$.

RESULTS

ROS FORMATION INDUCED BY TREATMENT WITH DIFFERENT CONCENTRATIONS OF GLUCOSE

As compared to 5.5 mM-glucose-treated cells (control), 30 mM-glucose-treated cells showed more accumulation of ROS from 1 day (1.7-fold increase) to 4 days (three-fold increase). However, ROS production was significantly inhibited in cells pre-treated with NAC at 4 days (Fig. 1A, $P < 0.05$). No significant differences were observed in ROS production between 24.5 mM-mannitol-treated cells and 5.5 mM-glucose-treated cells. Furthermore, ROS levels were markedly high (~2 fold) in Rosup-treated cells (Fig. 1A, $P < 0.05$).

DNA DAMAGE AND PARP1 ACTIVATION INDUCED BY TREATMENT WITH DIFFERENT CONCENTRATIONS OF GLUCOSE

DNA damage in 30 mM-glucose-HepG2 cells incubated for 2 days and 4 days was significantly higher (1.7-fold and 2.7-fold, respectively; $P < 0.05$) than in 5.5 mM-glucose treated cells. Interestingly, despite low ROS generation in the presence of Rosup, the DNA damage was slightly higher in Rosup-treated cells than in 30 mM-glucose-treated cells at 4days (Fig. 1B). This result suggested that glucose and Rosup caused oxidative damage by different mechanisms. To investigate whether DNA damage induced PARP1 activation, we determined the total poly(ADP-ribosyl)ated protein content by western blotting and presented the results as PARP1 activity. PARP1 activity in 30 mM-glucose-treated HepG2 cells incubated for 4 days was about two-fold more than that in control cells; this result was similar to the effect of treatment with 50 μg/ml Rosup and incubation for 15 min. Further, PARP1 activity was reduced in 30 mM-glucose-treated cells treated with 2.5 mM NAC for 30 min (Fig. 1C).

EFFECTS OF PARP1 ACTIVATION ON NAD LEVEL, AND NAD-DEPENDENT PROTEIN ACTIVITY UNDER DIFFERENT CONCENTRATIONS OF GLUCOSE

Dose-dependent suppression of PARP1 activity was observed in HepG2 cells treated with 30 mM glucose plus 0.3 μM–3 μM of PJ34 and incubated for 4 days. However, PJ34 had no effect on the expression of PARP1 protein (Fig. 1D). As expected, 30 mM-glucose-treated cells exhibited PARP1 activation and 50% decrease in cellular NAD level. After co culturing with 3 μM PJ34, the reduced NAD levels were restored by 75% (Fig. 2A, $P < 0.05$). Similar results were obtained for NAD-dependent protein SIRT1. Both the expression and activity of SIRT1 declined under high glucose concentration, and this effect was reversed by treatment with 1 μM and 3 μM of PJ34 (Fig. 2B and C, $P < 0.05$). Furthermore, we evaluated the effect of oxidative stress on SIRT1 expression. NAC restored the level of SIRT1 protein, which was attenuated by treatment with 30 mM glucose, whereas Rosup markedly decreased SIRT1 expression (Fig. 1C). Similar to SIRT1, AMPK activity (phosphorylation of AMPK) was inhibited by 30 mM glucose and this effect was reversed by treatment with 1 μM and 3 μM of PJ34 (Fig. 2D, $P < 0.05$). The NAD level (Fig. 2A) and the expression of SIRT1 and p-AMPK (Fig. 2C and D) were not affected by a low dose (0.3 μM) of PJ34. Taken together, our results suggest that PARP1 activation markedly influenced intracellular NAD levels, and the depletion of NAD induced downstream changes in SIRT1 and AMPK activity.

EFFECTS OF PARP1 ACTIVATION ON INSULIN SENSITIVITY UNDER DIFFERENT CONCENTRATIONS OF GLUCOSE

To determine whether PARP1 activation affected insulin signaling pathway and contributed to insulin sensitivity, we compared the levels of insulin-stimulated phosphorylated InsR, phosphorylated Akt, and phosphorylated GSK in cells treated with 5.5 mM or 30 mM glucose without or with PJ34 at different concentrations. As shown in Figure 3, in response to insulin stimulation, the phosphorylation of InsR at Tyr1150/1151 (p-Tyr1150/1151-InsR), Akt at Ser473 (p-Ser473-Akt) and GSK at Ser9 (p-Ser9-GSK) was impaired in 30 mM-glucose-treated cells. The combination of 30 mM glucose with PJ34 significantly reversed insulin-stimulated p-Tyr1150/1151-InsR, p-Ser473-Akt and p-Ser9-GSK expression (Fig. 3, $P < 0.05$).

EFFECTS OF PARP1 SIRNA ON INTRACELLULAR ACTIVATION OF PARP1, NAD CONTENT, AND INSULIN SENSITIVITY

PARP1 expression was suppressed after treatment with PARP1 siRNA, and the loss of activity was confirmed by Western blotting

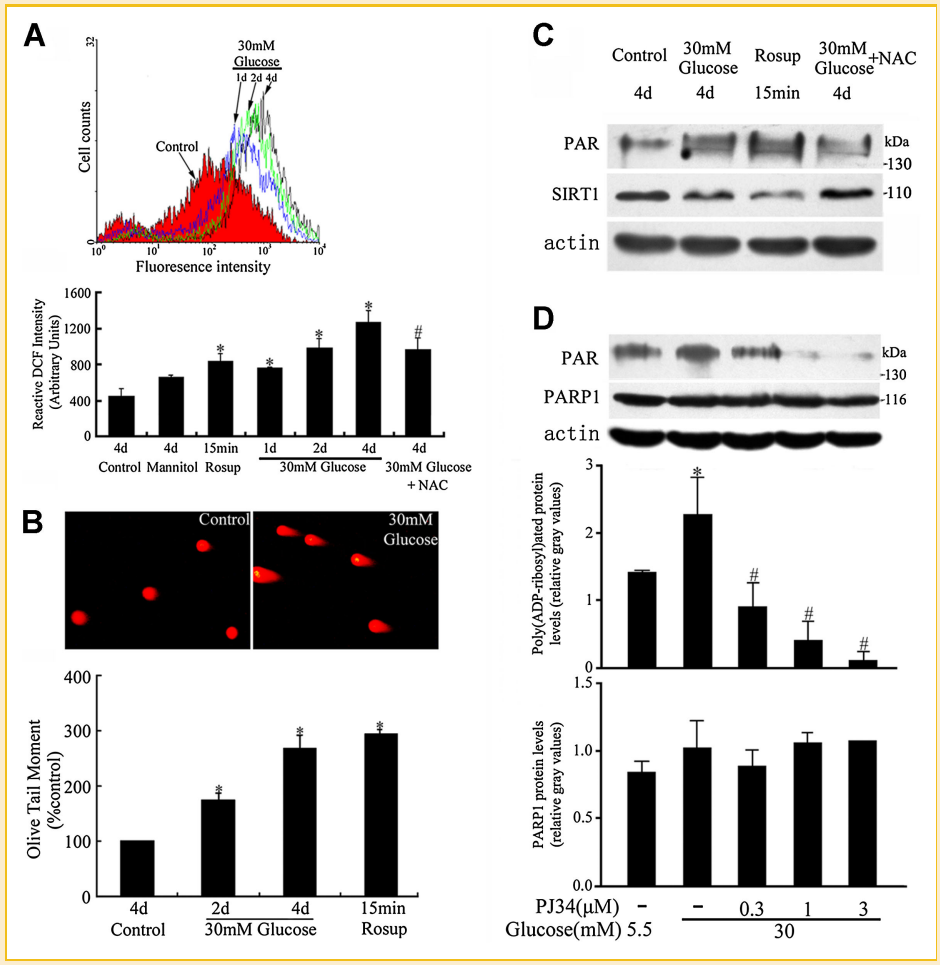

Fig. 1. ROS generation, DNA damage, and PARP1 activation induced by treatment with different concentrations of glucose. HepG2 cells were maintained at 5.5 mM glucose (control), 50 μg/ml Rosup, 30 mM glucose, or costimulated with 30 mM glucose and 2.5 mM NAC for the indicated period of time. 5.5 mM glucose plus 24.5 mM Mannitol was used as an osmotic control. ROS production was estimated fluorometrically by flow cytometry, and was expressed as DCF fluorescent intensity (A). Comet tail was observed under fluorescence microscope and DNA damage assessed by olive tail moment using comet assay software (B). The relationship of oxidative damage with PAR or SIRT1 expression was analyzed by Western blotting (C). HepG2 cells were cultured in 5.5 mM, 30 mM glucose with or without different concentrations of PARP1 inhibitor PJ34 (0.3 μM to 3 μM) for 4 days. PAR was suppressed by PJ34 in a dose-dependent manner ($P < 0.05$). The affect of PJ34 on expression of PARP1 was negligible (D). Actin was used as control to confirm equal loading of proteins. Bars represent the mean ± S.D. ($n = 3$). $^{*}P < 0.05$ versus control (A and B). $^{*}P < 0.05$ versus 5.5 mM glucose (D). $\#P < 0.05$ versus 30 mM glucose for 4 days (A and B). $\#P < 0.05$ versus 30 mM glucose without PJ34 (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 4A). Both PARP1 levels and activity were markedly down-regulated in 30 mM-glucose-treated HepG2 cells cultured with PARP1 siRNA, whereas there was no change in PARP1 level and activity in cells cultured with control siRNA. Intracellular NAD level and SIRT1 expression were reduced in 30 mM-glucose-treated cells, which were restored after transfection of cells with PARP1 siRNA (Fig. 4B and C, $P < 0.05$). Further, the phosphorylation of InsR in PARP1-siRNA-transfected cells was significantly higher as compared to that in the control-siRNA-transfected cells (Fig. 4D, $P < 0.05$). The results of transfection of HepG2 cells with PARP1 siRNA were similar to those obtained by treatment of the cells with PARP1 inhibitor PJ34.

EFFECTS OF PARP1 ACTIVATION ON HEPG2 CELL VIABILITY UNDER DIFFERENT CONCENTRATIONS OF GLUCOSE

PARP1 plays an important role in cell death; therefore, we analyzed the effect of PARP1 activation on the viability of glucose-treated HepG2 cells. As compared to 5.5 mM-glucose-treated cells, approximately 78% of 30 mM-glucose-treated cells survived after 4 days. PJ34 treatment prevented glucose toxicity in 30 mM-glucose-treated cells, and nearly 93% of the cells survived after treatment with 1 μM of PJ34 (Fig. 5, $P < 0.05$). This result indicated that high glucose concentration had slight effect on the viability of HepG2 cells and that PARP1 partially mediated this process.

DISCUSSION

The results of this study indicated that PARP1 is activated in response to elevated glucose metabolism accompanied by ROS generation and DNA oxidative damage. These changes, in turn, depleted intracellular NAD content, repressed SIRT1 and AMPK activity, and finally down-regulated insulin action (insulin insensitivity). Since type 2 diabetes is characterized by high

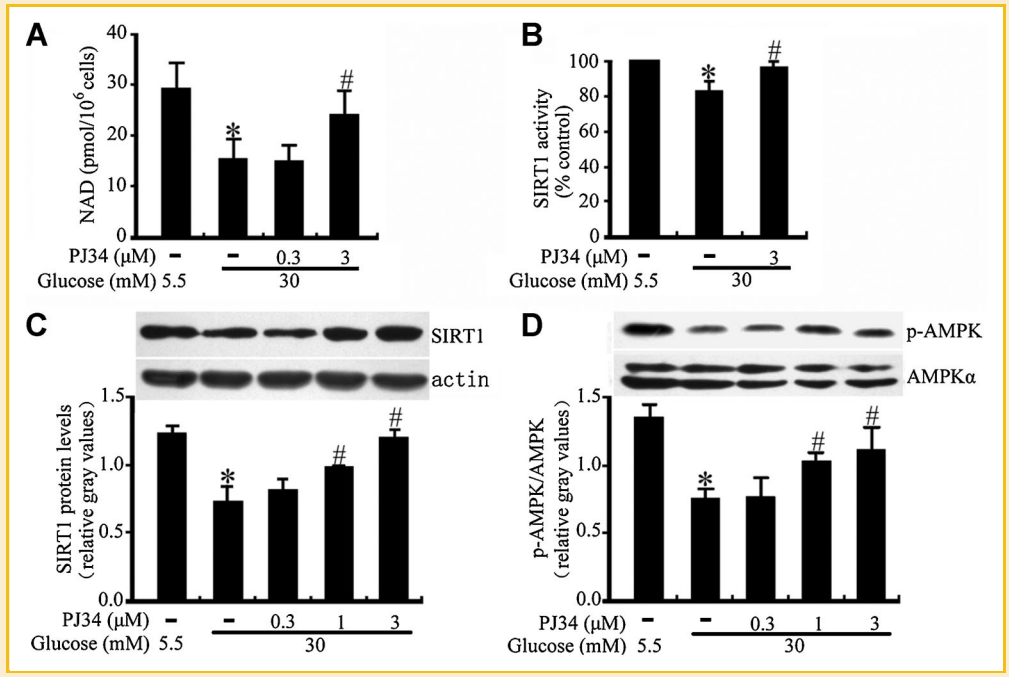

Fig. 2. Effects of PARP1 activation on NAD level and NAD-dependent protein activity under different concentrations of glucose. Exposure of HepG2 cells to 30 mM glucose for 4 d reduced intracellular NAD content (A), SIRT1 activity (B), SIRT1 expression (C), and AMPK activity (D, p-AMPK), however, all of these changes were reversed by co-cultured with PJ34 (A–D, $P < 0.05$). Actin and AMPKα were used as control to confirm equal loading of proteins. Bars represent the mean ± SD (n = 3). $^{*}P < 0.05$ versus 5.5 mM glucose. $\#P < 0.05$ versus 30 mM glucose without PJ34.

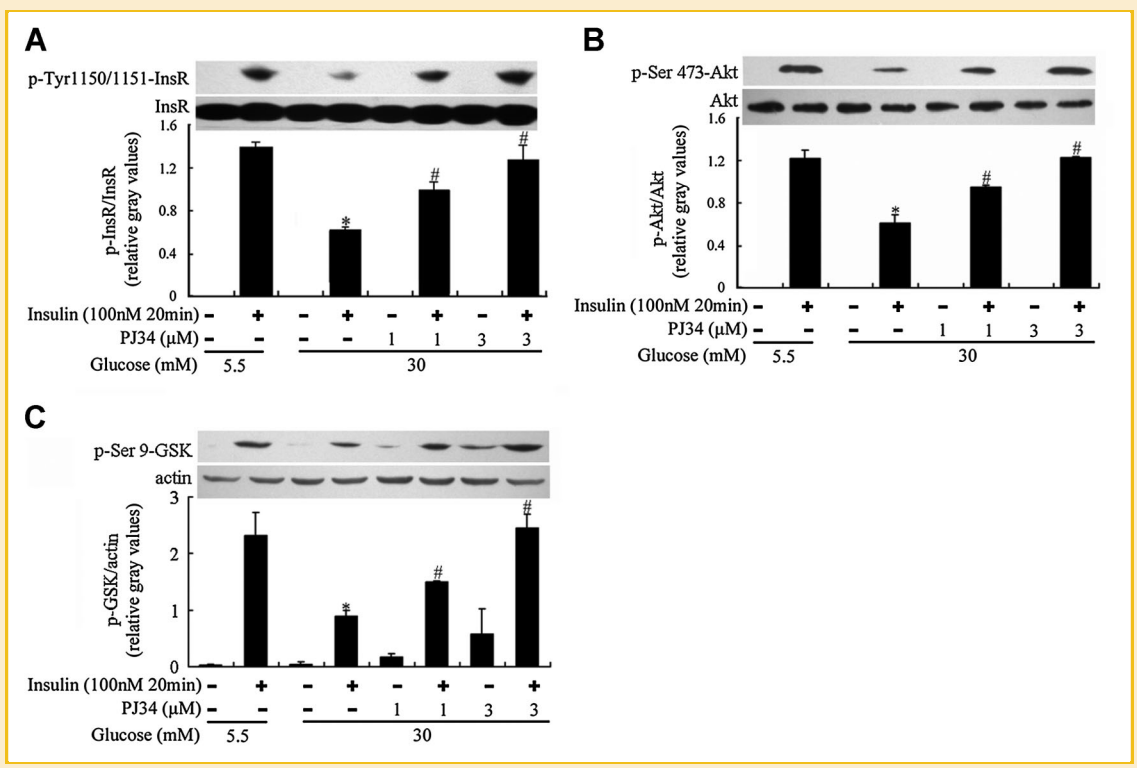

Fig. 3. Effects of PARP1 activation on insulin sensitivity under different concentrations of glucose. InsR phosphorylation (p-Tyr1150/1151-InsR), Akt phosphorylation (p-Ser473-Akt), and glycogen synthase kinase phosphorylation (p-Ser9-GSK) stimulated by insulin was impaired in HepG2 cells with 30 mM glucose for 4 days. Insulin sensitivity could be improved by adding PJ34 to the cells with 30 mM glucose ($P < 0.05$). Total InsR, Akt, and Actin was used as control to confirm equal loading of proteins. Bars represent the mean ± SD (n = 3). $^{*}P < 0.05$ versus 5.5 mM glucose. $\#P < 0.05$ versus 30 mM glucose without PJ34.

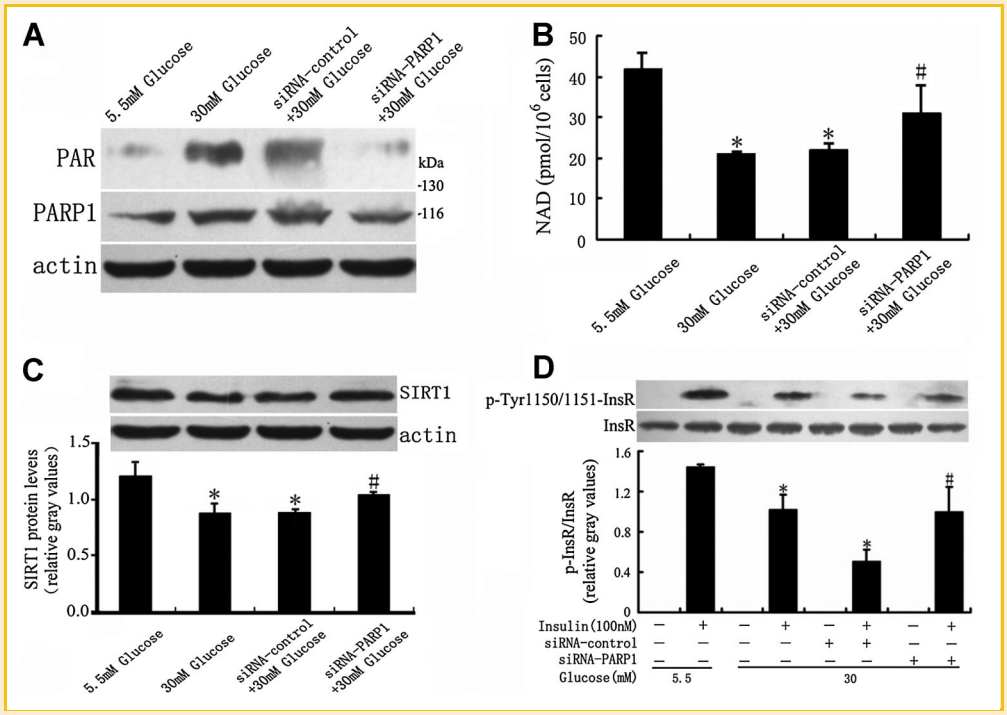

Fig. 4. Effects of PARP1 siRNA on intracellular activation of PARP1, NAD content, and insulin sensitivity. HepG2 cells were cultured in a medium containing 30 mM glucose for 4 days, and were transfected with siRNA-PARP1 on day 3. PAR and protein levels were detected by Western blot (A). NAD content (B), SIRT1 expression (C), and InsR phosphorylation (D) were reduced in siRNA-control group, and all of them were rescued by siRNA-PARP1 treatment ($P < 0.05$). Actin and total InsR were used as control to confirm equal loading of proteins. Bars represent the mean ± SD (n = 3). $^{*}P < 0.05$ versus 5.5 mM glucose. $\#P < 0.05$ versus siRNA-control.

extracellular glucose levels, our results may improve the understanding of the mechanisms by which chronic hyperglycemia influenced cell function in vivo.

A previous study suggested that PARP1 activation represented the two sides of a coin: on one hand it was a DNA repair enzyme and an indispensable factor for cell survival and on the other hand it caused depletion of cellular energy and lead to cell death [Virág and Szabó, 2002]. Herein, we showed that PARP1 activation was one of the pathways involved in the repression of SIRT1 and AMPK activity through the depletion of intracellular NAD. To investigate the effects induced by PARP1 activation, we used both PARP1 inhibitor PJ34 and PARP1 siRNA. Treatment with 1 and 3 μM of PJ34 protected intracellular NAD levels and improved AMPK phosphorylation and SIRT1 expression, which in turn reversed the insulin-stimulated p-Tyr1150/1151-InsR, p-Ser473-Akt and p-Ser9-GSK expression of 30 mM-glucose-treated HepG2 cells. And PJ34 did not affect p-Tyr1150/1151-InsR, p-Ser473-Akt and p-Ser9-GSK expression in 5.5 mM-glucose-treated cells (data not shown). The inhibition of Akt activation by PARP1, which involved in cell survival modulation, had also been elucidated in other papers [Tapodi et al., 2005; Szanto et al., 2009].

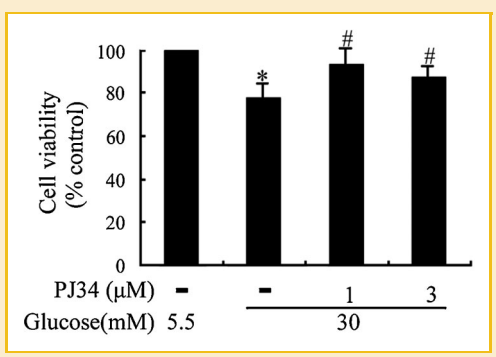

Fig. 5. Effects of PARP1 activation on HepG2 cell viability under different concentrations of glucose. HepG2 cells cultured in 30 mM glucose for 4 d induced cell counts reduced which could be restored by adding PJ34 ($P < 0.05$). Bars represent the mean ± SD (n = 3). $^{*}P < 0.05$ versus 5.5 mM glucose. $\#P < 0.05$ versus 30 mM glucose without PJ34.

PJ34 is a compound that targets the highly conserved catalytic domain of PARP enzymes. It is a non-specific pharmacological inhibitor of the activity of PARP1 as well as other PARP family proteins, such as PARP2 [Virág and Szabó, 2002]. To determine the specificity of this inhibitory effect of PJ34, we examined PARP1 inhibition after siRNA transfection. The effects of transfection of HepG2 cells with PARP1 siRNA were similar to those of the treatment of cells with the PARP inhibitor PJ34. Cellular NAD content, SIRT1 expression, and InsR phosphorylation were significantly higher in PARP1-siRNA-transfected cells as compared to those in control-siRNA-transfected cells. All the results of PARP1 siRNA transfection indicated that PARP1 played a significant role in modulating insulin sensitivity.

The RNA interference experiment suggested that strong inhibition of PARP1 could influence its roles in DNA repair [Tao et al.,

2009]. Therefore, we selected the condition with 50% inhibition of PARP1 protein in the experiment. Under this condition, PARP1 activity was significantly inhibited and all the effects of PJ34 treatment were also observed. Moreover, InsR phosphorylation in siRNA-transfected cells appeared to be lesser than that in untransfected cells. We observed that the siRNA transfection reagent, which inhibited the phosphorylation of InsR in HepG2 cells, had a non-specific effect (data not shown), and used the cells with non-specific transfection as controls (siRNA-control) in this experiment.

However, NAD depletion were only partially prevented by inhibiting PARP1; this result suggested that another mechanism may be responsible for the effects induced by enhanced glucose metabolism, such as the inhibition of NAD biosynthesis proteins NAMPT and NMNAT (data not shown).

In addition, our data showed that treatment with 1 and 3 μM PJ34 protected HepG2 cells from high-glucose-induced cell death, which was consistent with the findings of previous reports using other cell lines [Yu et al., 2002; Pillai et al., 2005]. Further, treatment with 3 μM PJ34 did not affect the viability of HepG2 cells cultured under normal glucose concentration (data not shown). This finding was inconsistent with the results of another report, in which treatment with 3 μM PJ34 affected HepG2 cell apoptosis [Huang et al., 2008]. This discrepancy can be attributed to the differences in detection methods and culture conditions used both the studies.

In the present study, we primarily discussed a novel pathway involved in glucose toxicity, which contributed to insulin insensitivity by mechanisms other than cell death through PARP1 activation related to NAD depletion and inhibition of SIRT1 expression. In addition to SIRT1, phosphorylation of AMPK was affected by PARP1 activity. Both the enzymes used in this study are well-known multifunctional enzymes involved in metabolic regulation [Towler and Hardie, 2007; Yamamoto et al., 2007; Yu and Auwerx, 2009] and both are regulated by metabolites [Rafaeloff-Phail et al., 2004; Revollo et al., 2004]. In this study, we found that PARP1-mediated decrease in NAD levels was related to the reduction of AMPK activity and decrease of SIRT1 expression by the same mechanism but to a different degree. As compared to the expression of AMPK phosphorylation, the expression of SIRT1, which is an NAD-dependent enzyme highly sensitive to NAD levels, was recovered to a greater degree by PJ34. Although the restoration of intracellular NAD level was not completely due to PJ34 treatment or PARP1 siRNA transfection, these treatments influenced SIRT1 expression and AMPK activation and played key roles in modulating insulin sensitivity [Longnus et al., 2005; Sun et al., 2007; Lin and Lin, 2008].

Our findings may improve the understanding of the potential role of PARP1 in glucose toxicity and insulin sensitivity. Metabolites, such as NAD, ATP, and so on, are not only the sources of cellular energy but also the modulators of cell recognition and cell response to changes in environment. Our results provide an evidence to suggest that the processes of metabolism, oxidation stress, and DNA damage are interlinked and regulated at an intracellular level. All the pathways had a common component, namely, NAD, which is a fundamental metabolite, whose importance has been highlighted by Imai [2009] as "NAD world".

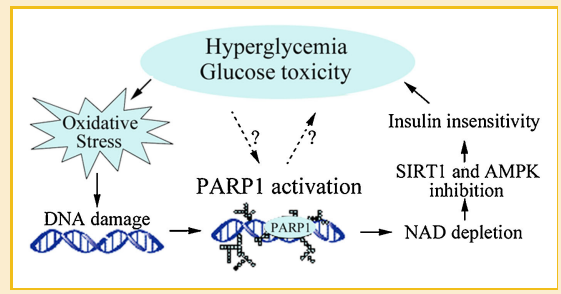

Fig. 6. Schematic model of PARP1 activation and downstream events leading to metabolic disorders under hyperglycemia. Solid line was the pathway involved in glucose toxicity proposed in this paper; dotted line indicated some possible pathway need exploring. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In conclusion, we investigated the role of PARP1 activation in glucose toxicity and found that PARP1-induced depletion of intracellular NAD level is partly responsible for the suppression of SIRT1 and AMPK activity, ultimately leading to insulin insensitivity (Fig. 6). The proposed pathway may be considered as a diabetic pathophysiologic process and can be targeted for pharmaceutical research.

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