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Trabajo Original

Obesidad y síndrome metabólico

Protective effect of manganese treatment on insulin resistance in HepG2 hepatocytes

Efecto protector del tratamiento con manganeso sobre la resistencia a la insulina en hepatocitos HepG2

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Abstract

Objectives: manganese (Mn) is closely related to type 2 diabetes *mellitus* and insulin resistance (IR), but the exact mechanism is unclear. This study aimed to explore the regulatory effects and mechanism of Mn on IR using hepatocyte IR model induced by high palmitate (PA), high glucose (HG) or insulin.

Methods: HepG2 cells were exposed to PA (200 μM), HG (25 mM) or insulin (100 nM) respectively, alone or with 5 μM Mn for 24 hours. The expression of key proteins in insulin signaling pathway, intracellular glycogen content and glucose accumulation, reactive oxygen species (ROS) level and Mn superoxide dismutase (MnSOD) activity were detected.

Results: compared with control group, the expression of phosphorylated protein kinase B (Akt), glycogen synthase kinase-3β (GSK-3β) and forkhead box O1 (FOXO1) in the three IR groups was declined, and this decrease was reversed by Mn. The reduction of intracellular glycogen content and increase in glucose accumulation in IR groups were also inhibited by Mn. Additionally, the production of ROS was increased in IR models, compared with normal control group, while Mn reduced the excessive production of ROS induced by PA, HG or insulin. However, Mn did not alter the activity of MnSOD in the three IR models.

Conclusion: this study demonstrated that Mn treatment can improve IR in hepatocytes. The mechanism is probably by reducing the level of intracellular oxidative stress, enhancing the activity of Akt/GSK-3β/FOXO1 signal pathway, promoting glycogen synthesis, and inhibiting gluconeogenesis.

Keywords:

Insulin resistance. Type 2 diabetes. Manganese.

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Resumen

Objetivos: el manganeso (Mn) está estrechamente relacionado con la diabetes *mellitus* tipo 2 y la resistencia a la insulina (RI), pero el mecanismo exacto aún no está claro. Este estudio tuvo como objetivo explorar los efectos reguladores y el mecanismo del Mn sobre la RI utilizando un modelo de RI en hepatocitos inducido por palmitato alto (PA), glucosa alta (HG) o insulina.

Métodos: las células HepG2 se expusieron a PA (200 μM), HG (25 mM) o insulina (100 nM), solas o junto con 5 μM de Mn durante 24 horas. Se evaluó la expresión de proteínas clave en la vía de señalización de la insulina, el contenido intracelular de glucógeno y la acumulación de glucosa, el nivel de especies reactivas de oxígeno (ROS) y la actividad superóxido dismutasa del manganeso (MnSOD).

Resultados: en comparación con el grupo de control, la expresión de proteína quinasa B fosforilada (Akt), la glucógeno sintasa quinasa-3β (GSK-3β) y la proteína *forkhead box* O1 (FOXO1) en los tres grupos de RI se redujo, y esta disminución fue revertida por el Mn. La reducción del contenido de glucógeno intracelular y el aumento de la acumulación de glucosa en los grupos de RI también fueron inhibidos por el Mn. Además, la producción de ROS aumentó en los modelos de RI en comparación con el grupo de control normal. Mientras que el Mn redujo la producción excesiva de ROS inducida por PA, HG o insulina. Sin embargo, el Mn no alteró la actividad de la MnSOD en los tres modelos de RI.

Conclusión: este estudio demostró que el tratamiento con Mn puede mejorar la RI en hepatocitos. El mecanismo probablemente sea mediante la reducción del nivel de estrés oxidativo intracelular, mejorando la actividad de la vía de señalización Akt/GSK-3β/FOXO1, promoviendo la síntesis de glucógeno e inhibiendo la gluconeogénesis.

Palabras clave:

Resistencia a la insulina. Diabetes tipo 2. Manganeso.

INTRODUCTION

Insulin resistance (IR) is associated with the impairment of the biological response to insulin stimulation of key target tissues, especially liver, muscle, and adipose tissue, which is the major factor to the pathogenesis of type 2 diabetes *mellitus* (1). The environmental lifestyle-related factors such as high-free fatty acid (FFAs) diet, over-nutrition, nutritional imbalance etc., have been well recognized as the risk factors leading to a sharp increase of IR (2-4). Exploring the connection between dietary factors and IR has become a research hotspot in the field of diabetes.

Manganese (Mn) is one of the essential micronutrients in the human body, which exists mainly in the form of Mn metalloenzymes, such as Mn superoxide dismutase (MnSOD) (5-7). Mn is widely distributed in the liver and is involved in multiple biological functions of cells, including serving as a cofactor for many enzyme systems, participating in the metabolism of glucose and lipids, improving the immune function, etc. (5-7). Two recent prospective cohort studies (8) demonstrated that an appropriate increase in dietary Mn intake could reduce the risk of type 2 diabetes *mellitus.* The researchers speculated that the mechanism probably be that dietary Mn can increase the activity of MnSOD and reduce the oxidative stress. The *in vivo* studies showed that rats with dietary Mn-deficient had reduced insulin secretion and impaired glucose tolerance (9), while dietary treatment with Mn increase the MnSOD activity in diabetic fatty Rats (10) and high-fat diet-induced mice (11), improve glucose tolerance and enhance insulin secretion (11). According to the current researches, Mn indeed has the protective function against type 2 diabetes *mellitus* and improves IR. However, the specific mechanism of Mn on IR is still unclear, and there are few related studies.

Liver, as the target organ of insulin, is the main site where the body regulates glycogen synthesis and gluconeogenesis, maintaining the stability of blood glucose (12). When in the condition of high glucose (13), FFAs (14,15) and high insulin (16), or certain cytokines such as tumor necrosis factor-α (TNF-α) (17), the hepatocyte can be induced to develop IR. Oxidative stress is considered to be the pivotal common soil mechanism for various factors leading to IR (18-20). The increased production of reactive oxygen species (ROS) during various harmful stimuli triggers the activation of stress-sensitive serine/threonine kinase signaling pathways, leading to impaired activation of phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) signaling pathway, accompanied by decreased downstream glycogen synthesis and increased gluconeogenesis in the liver (14,20,21).

In the present study, HepG2 cells were induced by high concentration of palmitic acid or glucose or insulin to establish hepatocyte IR model. The expression of key enzymes for insulin signal regulation, glucose metabolism related molecules, intracellular ROS and MnSOD enzyme activity was analyzed to explore the role and specific mechanism of Mn in improving IR and glucose metabolism, expecting to provide new ideas for the nutritional prevention of IR.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

$MnCl_2 \bullet 4H_2O$ and palmitic acid were purchased from Sigma-Aldrich Company (St Louis, MO, USA). D-glucose and recombinant human insulin were purchased from Solarbio (Beijing, China). Glycogen Content Assay Kit was purchased from Solarbio (Beijing, China). Glucose oxidase assay kit was purchased from Applygen (Beijing, China). 2-(*N*-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxy-D-glucose (2-NBDG) was purchased from Invitrogen (Carlsbad, CA, USA). Reactive Oxygen Species Assay Kit was purchased from Beyotime Biotechnology (Shanghai, China). SOD Assay kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). The antibodies against Akt and *p*-Akt (Ser473), GSK-3β and *p*-GSK-3β (Ser9), FOXO1 and *p*-FOXO1 (Thr24) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-β-actin antibody was purchased from ABclonal (MA, USA).

CELLS CULTURE AND TREATMENT

Human hepatoma cell line HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) and 10 % (v/v) fetal bovine serum (FBS) (Genial, USA) in 5 % (v/v)

CO_2 at 37 °C humidified atmosphere. HepG2 cells were seeded in six-well plates at a density of 5×10^5 cells/well and grown at 37 °C overnight, then exposed to palmitate (PA, 200 μM), high glucose (HG, 25 mM) or insulin (Insulin, 100 nM) respectively alone for IR model induction (15, 22) or with Mn (5 μM, MnCl2) for 24 hours. Then, cells were stimulated with 100 nM insulin for ten minutes and then harvested for further analysis.

WESTERN BLOT

The total cell lysates were obtained using RIPA lysis buffer (Beyotime, China). Then the protein was denatured and separated by SDS-PAGE, and further transferred onto PVDF membrane (Merck Millipore, USA). After blocking with TBST (0.5 % Tween-20) containing 5 % (w/v) non-fat milk, the membranes were then incubated with specific primary antibodies: anti-Akt/*p*-Akt (1:1000), anti-GSK-3β/*p*-GSK-3β (1:1000), anti-FOXO1/*p*-FOXO1 (1:1000) and anti-β-actin (1:50000) at 4 °C overnight in blocking solution. Following three times of washed with TBST, the membranes were incubated with HRP-conjugated secondary antibodies (1:7500) (Promega, USA) at room temperature for one hour. Images were acquired and quantified using Alpha chemiluminescence gel imaging system FluorChem™ E (Protein Simple, Inc., USA).

DETERMINATION OF GLYCOGEN CONTENT

Glycogen content was measured by glycogen content assay kit according to the instruction manual provided with the kit. Glycogen content was calculated and normalized with cellular protein content.

DETERMINATION OF INTRACELLULAR GLUCOSE LEVEL

The intracellular glucose was measured according to the instruction manual provided with the glucose oxidase assay kit. The glucose concentration was normalized with cellular protein concentration.

ROS DETECTION

HepG2 cells were incubated with DCFH-DA (2,000 μM) in serum-free medium at 37 °C for 20 minutes. Then cells were transferred into the centrifuge tube and centrifuged at 1,000 rpm for three minutes. The cell pellets were resuspended twice with PBS and filtered in a flow tube. The intracellular fluorescence intensity was measured by flow cytometry.

MnSOD ACTIVITY ASSAY

The activity of MnSOD was determined according to the instruction manual provided with the kit. The absorbance value was measured at the 450 nm wavelength. MnSOD activity was normalized with cellular protein concentration.

STATISTICAL ANALYSIS

Statistical analyses were performed using the SPSS 21.0 soft package. One-way ANOVA followed by Turkey's test was used to determine differences between groups, data were given as the means ± standard deviations (SD), p-values < 0.05 and < 0.01 were considered as significant.

RESULTS

Mn REGULATES THE INSULIN SIGNALING PATHWAY BY INCREASING THE ACTIVATION OF Akt

Akt is a key kinase in the PI3K/Akt pathway of the insulin signaling pathway (23). The phosphorylation and activation of Akt is critical for the activity of downstream glucose-metabolizing enzymes. As shown in figure 1, the expression of *p*-Akt in all three IR model groups was more significantly inhibited, compared with the normal control group, suggesting that the IR models were successfully established. Moreover, Mn treatment obviously enhanced the phosphorylation of Akt, and reversed the inhibitory effect of high concentrations of PA, glucose or insulin on Akt phosphorylation (Fig. 1).

Mn TREATMENT IMPROVES GLUCOSE METABOLISM DISORDER IN IR HepG2 CELLS

Mn treatment improves glycogen synthesis ability of IR hepatocytes

Glycogen synthase kinase-3β (GSK-3β) is a kinase downstream of Akt that is responsible for glycogen synthesis (24). As shown in figure 2, the expression of *p*-GSK-3β in all three IR models was more significantly decreased, compared with the normal control group. While Mn treatment obviously increased the expression of *p*-GSK-3β, it reversed the inhibition of GSK-3β phosphorylation induced by PA, HG or insulin (Fig. 2A-C). To further identify the role of Mn on glycogen synthesis, intracellular glycogen content was investigated. The similar results were obtained that three IR models all clearly reduced intracellular glycogen content, and Mn treatment significantly increased intracellular glycogen content, compared with three IR model groups (Fig. 2D-F).

Mn treatment reduces gluconeogenesis ability of IR hepatocytes

Forkhead box O1 (FOXO1) is a transcription factor involved in the regulation of hepatic gluconeogenesis and is also regulated

by Akt phosphorylation (24). The results indicated that *p*-FOXO1 protein expression levels were all significantly decreased in three IR models, compared with the normal control group (Fig. 3). While Mn treatment upregulated the phosphorylation of FOXO1, it reversed the inhibition effect of FOXO1 phosphorylation induced by PA, HG or insulin.

Mn treatment reduces intracellular glucose accumulation of IR hepatocytes

To further verify the positive effect of Mn on glucose metabolism in hepatocytes, intracellular glucose net content was detected. Results showed that the intracellular glucose contents were all remarkably increased in three IR model groups, compared with normal control group, and Mn treatment decreased the intracellular glucose content of three IR model groups (Fig. 4). However, this positive effect of manganese on glucose accumulation in hepatocytes was not observed in the cells supplemented with manganese alone.

Mn TREATMENT CAN REDUCE INTRACELLULAR ROS LEVELS

Due to the fact that the increase of ROS production can activate a variety of stress-sensitive signaling pathways and impair the normal function of insulin signals (20,21), the intracellular ROS levels were measured by flow cytometry to observe the effects of Mn treatment on oxidative stress. Figure 5 shows that intracellular ROS levels were significantly increased in three IR model groups compared with the control group. As expected, Mn treatment remarkably prevented the production of ROS induced by high concentration of PA, glucose or insulin.

THE ACTIVITY OF MnSOD IN Mn TREATMENT CELLS WAS UNCHANGED

To further explore how Mn treatment affects intracellular ROS, the antioxidant enzyme activity of MnSOD was detected. However, as shown in figure 6, there were no differences in MnSOD enzyme activity in the three IR model groups, compared with the normal control group. Moreover, Mn treatment did not affect the activity of MnSOD enzyme, compared with model groups.

DISCUSSION

This study demonstrated that Mn treatment could ameliorate hepatocyte IR and glycometabolic disorder, with the molecular mechanism of inhibiting the ROS production and activating the Akt/GSK3β/FOXO1 signaling pathway. This is the first demonstration that Mn treatment improves the hepatic HepG2 IR and glucose metabolism disorder induced by high PA, HG, or high insulin.

There are several high-risk factors that contribute to the development of IR, including increased circulating fuels, glucose and FFAs. Researches also showed that the *in vitro* IR model can be induced by high glucose, high FFAs, and high insulin to simulate the diabetic conditions of the body (14,22,25). In the present study, we established IR models with PA or glucose or insulin respectively in HepG2 cells. Results showed that the expression of *p*-Akt was significantly downregulated when HepG2 cells exposed with high concentration of PA, glucose or insulin, indicating that three different hepatocyte IR models were successfully established. Meanwhile, detection of the net content of intracellular glucose were all increased in three IR models, which further supported the successful establishment of hepatocyte IR model. While inducing IR, Mn was added to observe the preventive effect on IR.

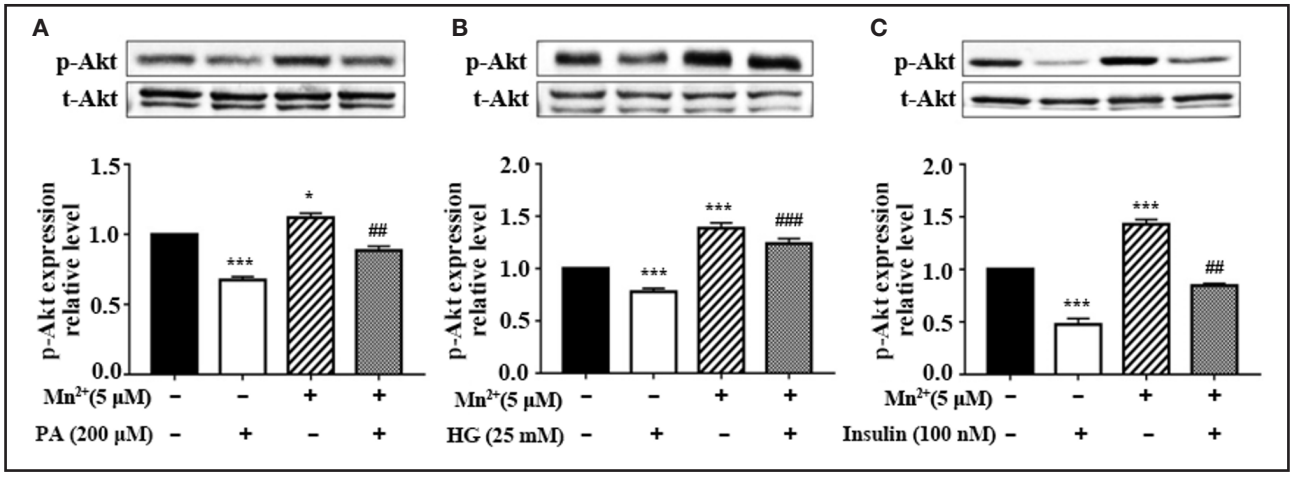

Figure 1.

Effects of Mn treatment on insulin signaling transmit in hepatocytes. A. Protein expression of *p*-Akt in PA-induced HepG2 cells. B. Protein expression of *p*-Akt in HG-induced HepG2 cells. C. Protein expression of *p*-Akt in insulin-induced HepG2 cells. $^{*}p < 0.05$ and $^{***}p < 0.001$ *vs* normal control group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* model group.

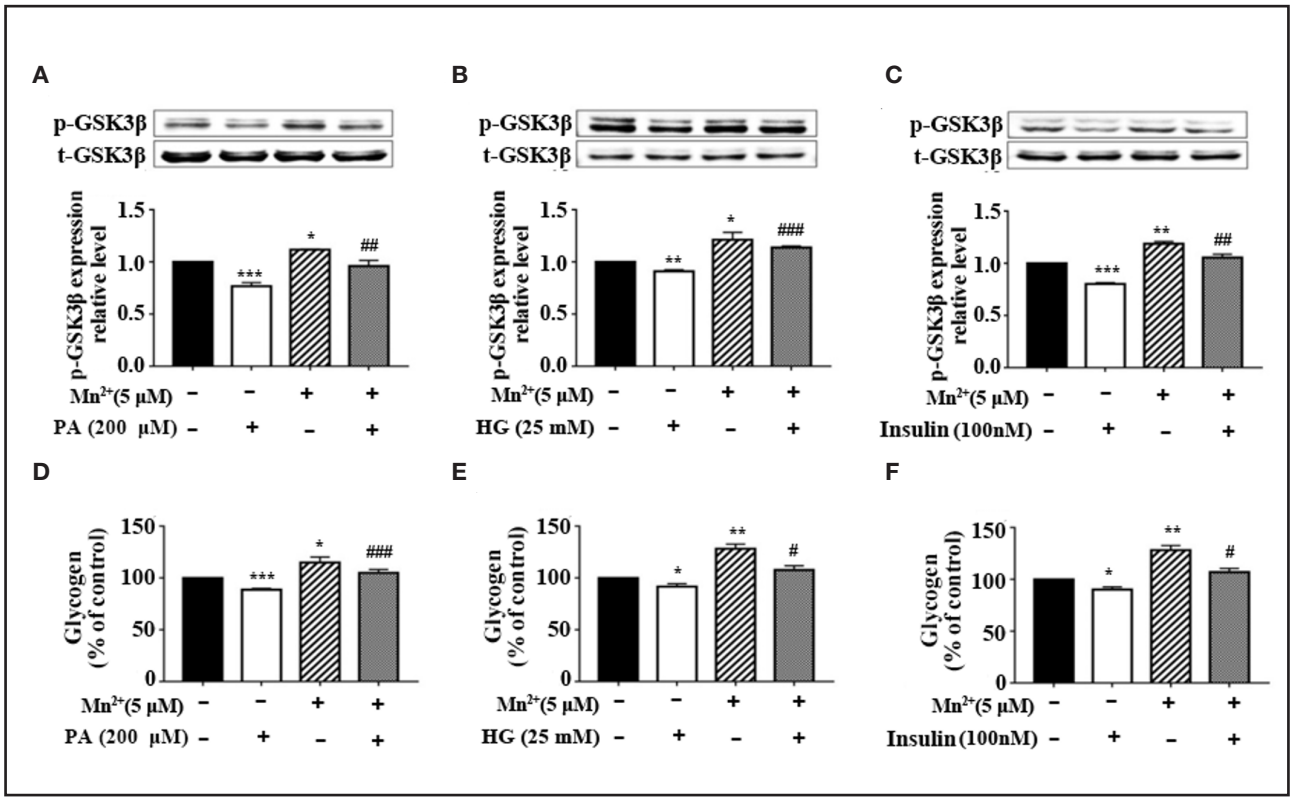

Figure 2.

Effects of Mn treatment on glycogen synthesis in hepatocytes. A-C. Protein expression levels of *p*-GSK3β in HepG2 IR models. D-F. The intracellular glycogen content in HepG2 IR models. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* normal control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* model group.

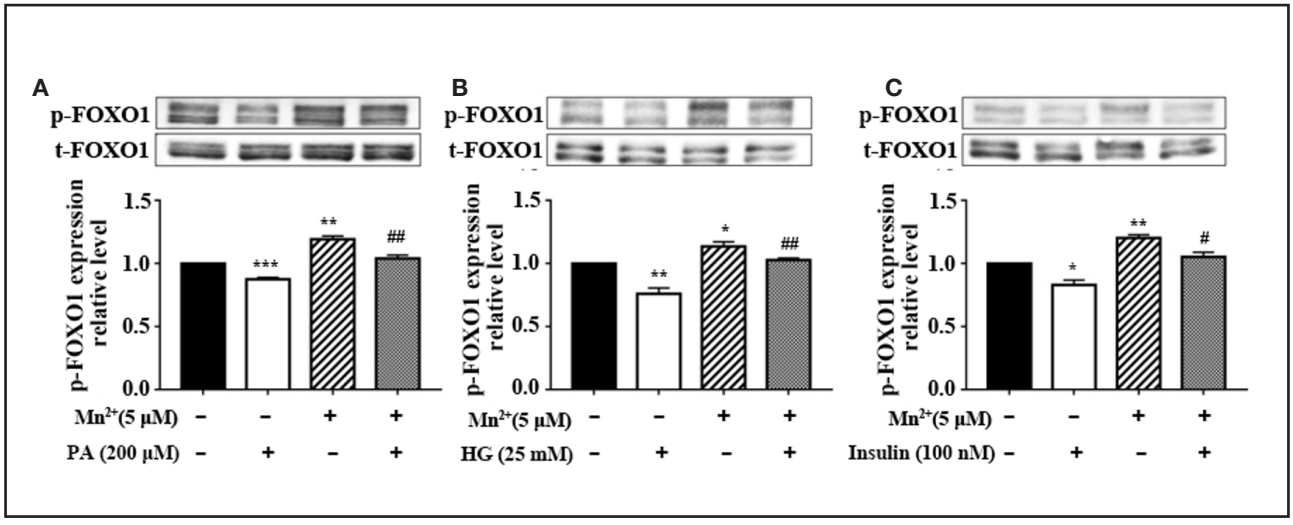

Figure 3.

Effects of Mn treatment on gluconeogenesis in hepatocytes. A. Protein expression levels of *p*-FOXO1 in PA-induced HepG2 cells. B. Protein expression levels of *p*-FOXO1 in HG-induced. C. Protein expression levels of *p*-FOXO1 in insulin-induced HepG2 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* normal control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs* model group.

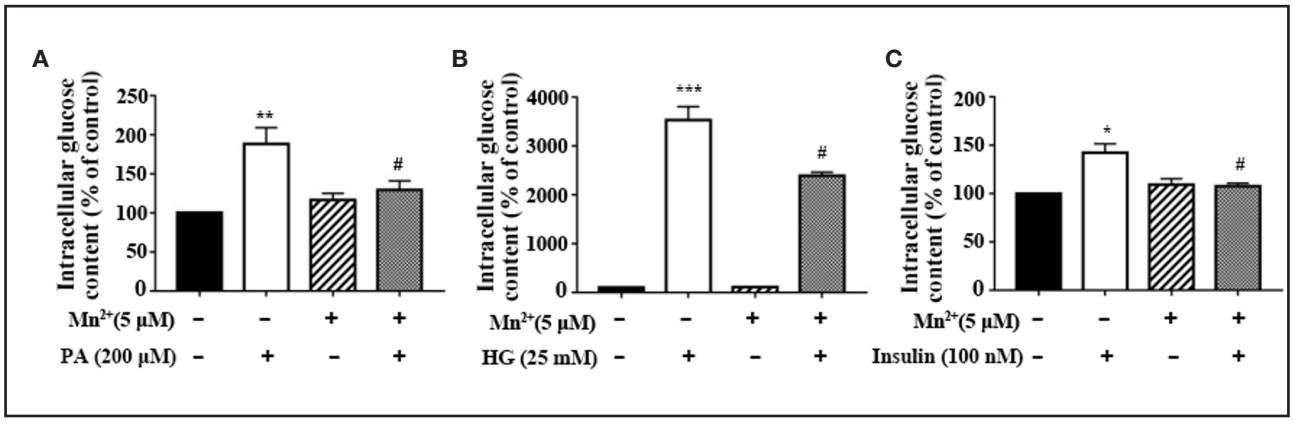

Figure 4.

Effects of Mn treatment on the net glucose net in hepatocytes. A. Relative levels of intracellular glucose content in PA-induced HepG2 cells. B. Relative levels of intracellular glucose content in HG-induced HepG2 cells. C. Relative levels of intracellular glucose content in insulin-induced HepG2 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* normal control group; $^{\#}p < 0.05$ *vs* model group.

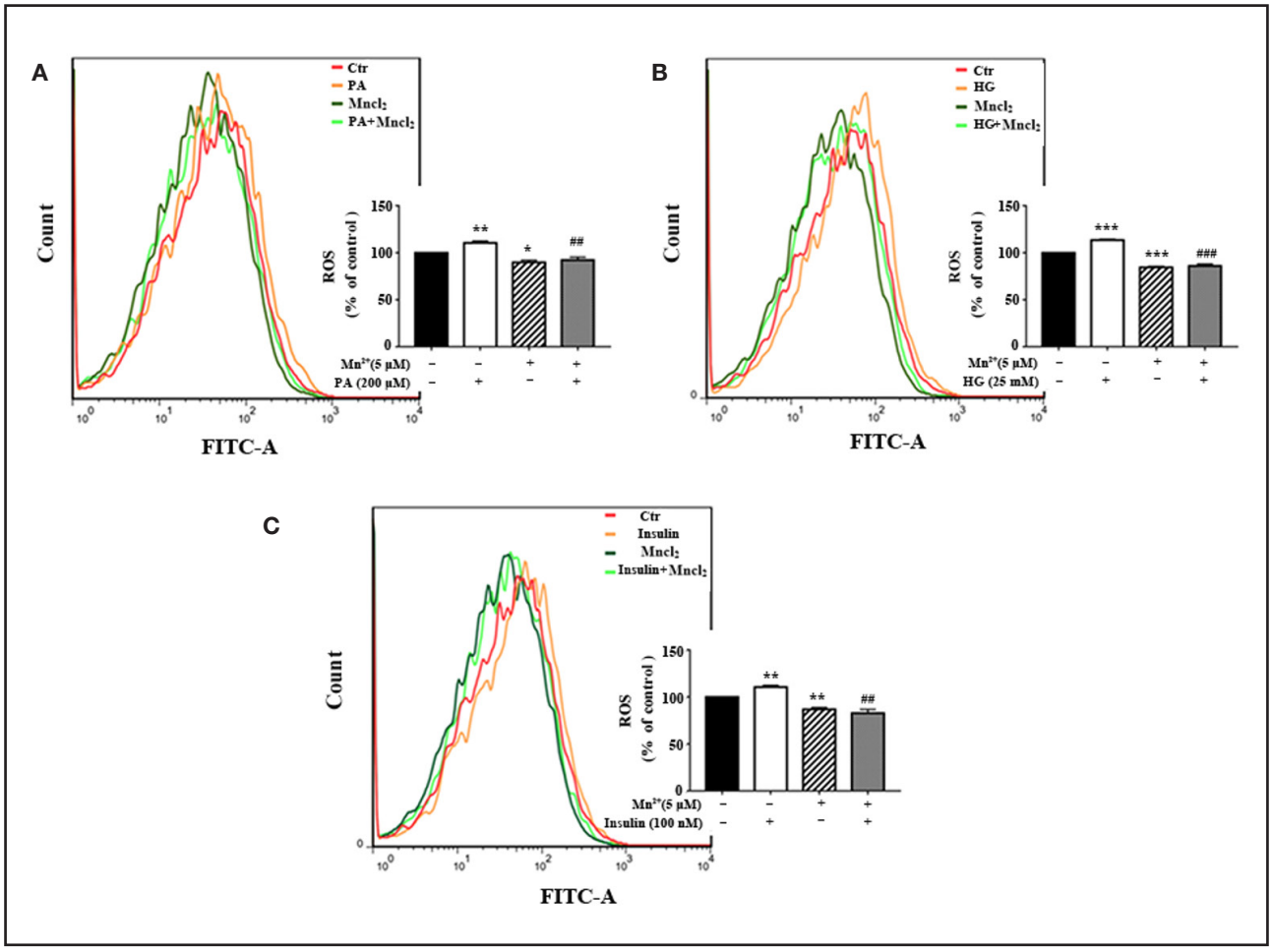

Figure 5.

Effects of Mn treatment on ROS levels in hepatocytes. A. Relative levels of ROS in PA-induced HepG2 cells. B. Relative levels of ROS in HG-induced HepG2 cells. C. Relative levels of ROS in insulin-induced HepG2 cells. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* normal control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs* model group.

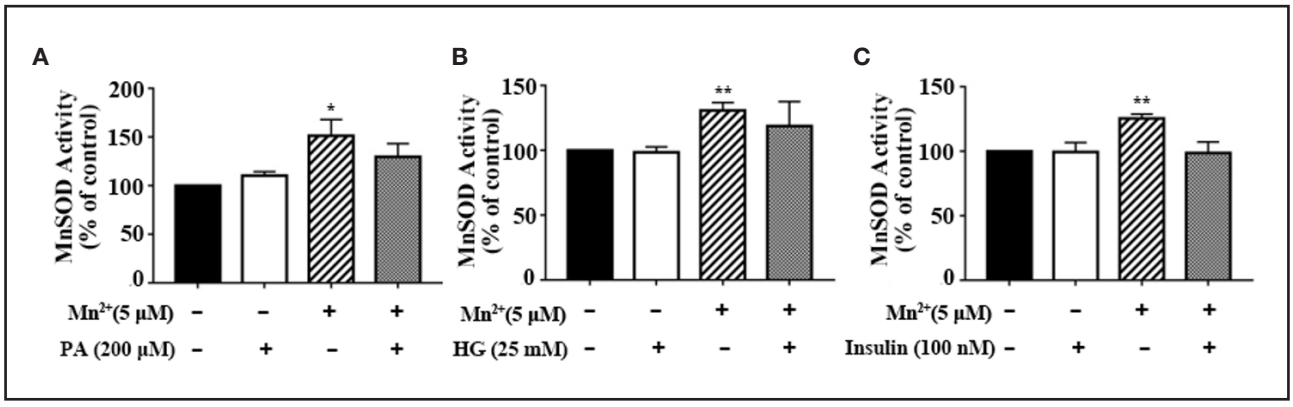

Figure 6.

Effects of Mn treatment on the activity of MnSOD in hepatocytes. A. Relative levels of MnSOD activity in PA-induced HepG2 cells. B. Relative levels of MnSOD activity in HG-induced HepG2 cells. C. Relative levels of MnSOD activity in insulin-induced HepG2 cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs* normal control group.

The simultaneous addition of Mn and inducer simulates the antagonistic and preventive effects of Mn when the dietary Mn intake is high in real life. Currently, many studies on the prevention of IR by nutritional factors have been designed in this way (26,27). When Mn and inducer are added at the same time, there may be a direct interaction between them. Of course, this direct interaction is probably either a part of the mechanism of Mn to prevent IR, or will produce bias, which needs to be further explored in our follow-up work.

Dysfunction of insulin signal transduction pathway directly contributing to hepatic IR and glucose metabolism disorder, the PI3K/Akt signal is a key component of insulin signal transduction pathway (23). During IR, the expression of *p*-Akt is decreased and the insulin signal cannot be transmitted, leading to an incapacitation for the regulation of downstream glucose metabolism. Some studies have shown that certain nutrients, such as the phytochemicals like quercetin, and mulberry anthocyanin extract, can significantly improve high glucose- or high FFAs-induced IR by increasing the phosphorylation of Akt (15,28,29). Micronutrients like zinc can also improve the inhibition of Akt phosphorylation induced by high FFAs (27). Consistently, this study showed that Mn treatment could upregulate the expression levels of *p*-Akt induced by PA, HG or insulin, suggesting that Mn treatment can improve the insulin signal transmission disorder in hepatic IR via promoting the activation of PI3K/Akt signaling.

GSK-3β and FOXO1 are both the downstream of PI3K/Akt signaling pathway, and play important roles in regulating glucose metabolism disorder (24). By increasing glycogen synthesis and inhibiting hepatic glucose output, the liver contributes to the disposal of enteral glucose loads, thus helping to maintain normal glucose tolerance (30). GSK-3β is a serine/threonine kinase that regulates the activity of glycogen synthase in the liver (31). When the insulin signaling pathway is activated, GSK-3β will be phosphorylated and inactivated to activate glycogen synthase and increase glycogen content in the liver (32). The present study showed that the phosphorylation of GSK-3β were all decreased in three IR models, while Mn treatment reversed the inhibitory effect of GSK-3β phosphorylation in all IR models (Fig. 2A-C). Furthermore, Mn treatment increased the intracellular glycogen content of three model groups (Fig. 2D-E). These results indicated that Mn treatment improved the IR hepatocytes glycogen synthesis and increased the intracellular glycogen content. On the other hand, during fasting, the liver provides glucose through gluconeogenesis pathway to maintain normal blood glucose and ensure the normal function of cells (30). FOXO1 is a nuclear transcription factor that induces the transcriptional expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, two key rate-limiting enzymes in the gluconeogenesis pathway, which can increase blood glucose (33). Once being phosphorylated, FOXO1 will translocate from nucleus to cytoplasm, thus inhibiting its transcriptional activity and translational expression, causing the decrease of gluconeogenesis (34). The results in the present study indicated that the phosphorylation of FOXO1 were all decreased in three different IR models (Fig. 3). While Mn treatment upregulated the FOXO1 phosphorylation, it reversed the inhibitory effect of FOXO1 phosphorylation in three IR models (Fig. 3). These data demonstrated that Mn treatment inhibited the gluconeogenesis of IR hepatocytes. This work thus far suggested that Mn treatment could effectively improve the abnormal glycogen synthesis and gluconeogenesis in IR hepatocytes through Akt/GSK3β/FOXO1 signaling pathway.

Oxidative stress plays an important role in contributing to the development of IR and type 2 diabetes (20). Loh et al. showed that physiological ROS can promote the sensitivity of host to insulin (35), but long-term stimulation of high-glucose, high-FFAs or hyperinsulinemia could cause the excessive production of ROS through mitochondrial pathway, ultimately leading to IR (36,37). Studies have demonstrated that the excessive production of ROS could activate oxidative stress-sensitive pathways, resulting in decreased IRS-1/2 tyrosine phosphorylation, which in turn causes decreased phosphorylation of Akt and its downstream signaling (such as GSK3β and FOXO1), and eventually inducing

impaired glycogen synthesis and aggravated gluconeogenesis in liver (14, 38). In the present study, the production of ROS was increased in IR HepG2 cells. As expected, Mn treatment reduced the high-level of ROS in three IR models. Based on the results obtained, we speculated that the mechanism of Mn in improving the IR hepatocyte glucose metabolism disorder probably partly through the inhibition of ROS production.

MnSOD is one of the typical Mn-dependent metalloenzymes, which is an important antioxidant enzyme in mitochondria. MnSOD has the activity of eliminating excessive ROS and reducing oxidative stress (16). However, the present study showed that Mn treatment did not affect the MnSOD activity in all three IR HepG2 cells, suggesting that the regulatory effects of Mn on ROS involved other mechanisms independent of MnSOD. Similar to our data, previous studies have shown that Mn treatment decreased the production of ROS in the liver and ameliorated endothelial cell dysfunction mediated by adiponectin, independent of MnSOD (10,11). Consequently, further studies are needed to determinate the specific mechanisms of Mn alleviating oxidative stress, improving hepatocyte IR and glucose metabolism. Certain studies have shown that miRNAs are involved in oxidative stress, IR regulation, and regulating insulin metabolic activities, including AKT, GSK-3β, etc. (39,40). Zheng et al. demonstrated that miR-195 was upregulated in the H2O2-induced oxidative stress cell model or hepatic tissue and retina tissue of STZ-induced diabetic rats and silencing of miR-195 significantly reduced ROS level in the heart of diabetic mice (41). Moreover, miR-1 (42) and miR-200c (43) have also been proved to be involved in regulating oxidative stress. Therefore, the miRNA regulation mechanism provides us with a new research perspective, which probably the specific regulation mechanism of Mn supplementation on glucose metabolism and oxidative stress.

Taken together, our work demonstrated for the first time that Mn treatment can improve IR induced by PA, HG or insulin in HepG2 hepatocytes, and revealed that the potential mechanisms are associated with the inhibition of ROS production and activation of Akt/GSK3β/FoxO1 signaling. These data suggested that proper supplement of dietary Mn may be an effective way to prevent IR, which provides new ideas and certain research basis for the research and treatment of type 2 diabetes *mellitus.*

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