

High-dose insulin inhibits gap junction intercellular communication in vascular smooth muscle cells

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Received May 15, 2014; Accepted January 30, 2015

DOI: 10.3892/mmr.2015.3437

Abstract. Gap junction intercellular communication (GJIC) is important in mediating intercellular substance and signal transmission. Connexin (Cx)43 is a major component involved in GJIC in vascular tissue and its abnormal expression is closely associated with various vascular diseases. Insulin resistance is the central component of metabolic syndrome, and high doses of insulin can affect vascular function through multiple pathways, resulting in cardiovascular disease. However, the effects of insulin on GJIC function and connexin (Cx)43 expression in vascular smooth muscle cells (VSMCs) remain unclear. Following treatment of VSMCs with different doses of insulin, a fluorescence recovery after photobleaching (FRAP) assay was performed to evaluate GJIC function in treated VSMCs. The results showed that high-dose insulin suppressed GJIC function. Western blot assays further demonstrated that high-dose insulin induced the phosphorylation of Cx43 at s368 and downregulated the expression of Cx43. H_2O_2 release assays demonstrated that high-dose insulin treatment significantly elevated the cellular H_2O_2 level. In addition, compared with cells treated with high-dose insulin, pretreatment with catalase significantly restored the cellular GJIC function, decreased the phosphorylation level of Cx43 at s368, and enhanced Cx43 expression. In conclusion, these data indicate that high-dose insulin inhibits cellular GJIC function through the oxidative stress-activated signaling pathway. This phenomenon may also constitute a potential mechanism underlying the pathogenesis of insulin resistance and its complications.

Introduction

Gap junctions are membrane channel structures between neighboring cells that mediate gap junction intercellular communication (GJIC), which is an important pathway involved in intercellular substance and signal transmission. GJIC occurs throughout vascular tissues and is pivotal in the maintenance of normal vascular function (1-3) and the repair of vascular damage (4,5). The basic structural unit of the gap junction is connexin. The major types of connexins expressed in vascular tissue include Cx43, Cx40, Cx37 and Cx45 (6), with Cx43 exhibiting the highest level of expression in this tissue (7,8). The abnormal expression or dysfunction of Cx43 can affect the proliferation, migration and differentiation of vascular smooth muscle cells (VSMCs) (9-11). In addition, Cx43 participates in the pathophysiological processes of vasoconstriction (12-14), coronary artery spasm (15,16) and atherosclerosis (17-19).

Insulin is an important hormone involved in metabolic regulation and is required for the maintenance of normal blood vessel function (20). In insulin-resistant states, high levels of insulin in the blood are closely associated with various cardiovascular diseases (21-23). However, the involvement of insulin in the pathogenesis of cardiovascular diseases through its effects on connexin and GJIC remains unclear. In this study, by treating VSMCs with different doses of insulin, the effects of insulin on GJIC, Cx43 phosphorylation, and Cx43 expression were explored under physiological and pathological conditions (presence of 1 or 100 nmol/l insulin, respectively). Furthermore, the possible mechanisms underlying these effects of insulin were investigated. The aim of this study was to clarify the molecular mechanisms that contribute to vascular dysfunction in conditions associated with insulin resistance.

Materials and methods

Cell culture. All animal treatment procedures were performed in accordance with the provisions of the Institutional Animal Ethics Committee of China Medical University. The present study was approved by the ethics committee of China Medical University (Shenyang, China). Eight-week-old healthy Sprague-Dawley rats were obtained from the Experimental Animal Center of China Medical University (Shenyang, China). The rats were housed under specific pathogen-free conditions ($23\pm 2^\circ\text{C}$, $60\pm 5\%$ humidity, 12:12 h light:dark cycle) and allowed free access to food and water. The rats were sacrificed by ether

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Key words: insulin, vascular smooth muscle cell, gap junction intercellular communication, connexin 43

inhalation after 1 week and their thoracic aortas were harvested under sterile conditions. As described previously (24), primary VSMCs were dissociated. Briefly, the thoracic aortas were washed in pre-cooled phosphate-buffered saline (PBS), and the adventitia and intima were removed. Subsequently, the reserved media tunica was transferred to a culture flask, cut into ~1 mm³ sections, stuck to the bottom and the bubbles were pressed out by tweezers. The flask was gently flipped upside down and 5-6 ml Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) was added. The culture flask was cultured in an incubator containing 5% CO₂ and 95% air, at 37°C. After 4 h, the flask was returned to an upright position and the tissue was completely immersed in the culture medium for 5-7 days. The VSMCs that had grown out from the edges of the tissue blocks were digested using 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China), seeded in a new flask and cultured in DMEM containing 10% FBS, 2 mmol glutamate (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), 100 U/ml penicillin (Beijing Solarbio Science & Technology Co., Ltd.) and 100 µg/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) at 37°C in a cell culture incubator with 5% CO₂. The purity of cultured VSMCs was determined using immunocytochemical staining with a monoclonal antibody specific for α-actin.

Immunocytochemistry. Briefly, VSMCs were grown to subconfluence on coverslips, fixed in 4% paraformaldehyde (Sinopharm Chemical Reagent, Shanghai, China) and permeabilized with 0.5% Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd.). Following treatment with 3% hydrogen peroxide (Sinopharm Chemical Reagent) and 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.), the coverslips were incubated overnight at 4°C with a mouse monoclonal antibody targeting α-actin (1:300 dilution; cat. no. sc-58669; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The coverslips were then incubated at 37°C for 40 min with a biotinylated goat anti-mouse immunoglobulin G antibody (cat. no. A0286; Beyotime Institute of Biotechnology). Immunostaining was detected using streptavidin-horseradish peroxidase conjugate (Beyotime Institute of Biotechnology) and visualized with 3, 3'-diaminobenzidine (Beyotime Institute of Biotechnology). Finally, the sections were counterstained with hematoxylin (Beijing Solarbio Science & Technology Co., Ltd.) and observed under a light microscope (DP73; Olympus, Tokyo, Japan).

Experimental grouping and determination of glucose uptake. VSMCs were inoculated into 24-well plates and cultured in a cell culture incubator. After 24 h in culture, 1 or 100 nmol/l insulin was added to the culture medium, followed by incubation for another 24 h. The glucose uptake of each group of cells was measured as described by Pyla *et al.* (25). Briefly, the cells were washed with Krebs-Ringer-HEPES buffer containing 15 mmol/l HEPES, 105 mmol/l NaCl, 5 mmol/l KCl, 1.4 mmol/l CaCl₂, 1 mmol/l KH₂PO₄, 1.4 mmol/l MgSO₄ and 10 mmol/l NaHCO₃, pH 7.4; followed by incubation in a 0.2 mmol/l 2-deoxy-D-glucose (2-DG) solution containing 1 µCi/ml 2-[³H] DG for 30 min. Subsequently, cells were washed three times with pre-cooled phosphate-buffered saline

and lysed with 0.4 mol/l NaOH. After neutralization with HCl, aliquots of cell lysate were used to determine glucose uptake with a liquid scintillation spectrometer (PerkinElmer Wallac, Inc., Gaithersburg, MD, USA).

Fluorescence recovery after photobleaching (FRAP) assay. FRAP experiments were conducted as previously described (26). After VSMCs grew to 80% confluence, different concentrations of insulin (0, 1 or 100 nmol/l) were added to the culture medium. After 24 h, the culture medium was discarded, and the cells were treated with 6-carboxyfluorescein diacetate (6-CFDA, Invitrogen Life Technologies, Carlsbad, CA, USA) at a final concentration of 10 µmol/l for 20 min. 6-CFDA can easily pass through cell membranes and decompose into 6-CF, which cannot pass through cell membranes, but can spread through gap junctions (27). The excitation wavelength of 6-CF is 490 nm, and cells labeled with it can be observed as green under a fluorescent microscope. After washing twice with D-Hank's solution, the cells were observed under a laser-scanning confocal microscope (FV1000S-SIM/IX81; Olympus).

Three types of cells were selected for the FRAP assay. The first type included cells that tightly connected with neighboring cells and demonstrated GJIC. The fluorescence recovery after photobleaching of these cells reflected the level of GJIC. The second type included isolated cells that did not demonstrate GJIC with neighboring cells and thus were used as a self-control in this experiment. The third type included cells that were tightly connected with neighboring cells and showed spontaneous photobleaching. This type of cells did not require photobleaching and thus were used for background correction. Photobleaching was conducted with a laser power of 500 mW, duration of the photobleaching pulse of 200 msec, a bleaching rate of 30-60%, a bleaching intensity of 100%, and a scanning intensity of 20%. After computer-assisted precise positioning, intracellular fluorescence in selected cells was bleached with a laser beam. The bleached cell was then monitored, and images were acquired (FV1000 confocal operations software; Olympus) continuously every 10 sec for 5 min for the detection of fluorescence recovery. The maximum fluorescence recovery proportion and the fluorescence recovery rate were used as indicators for the evaluation of GJIC in each group. The fluorescence recovery rate after photobleaching was calculated with the following formula: $[(I_t - I_0)/(I - I_0) \times 100\%]/t$, where I_t is the relative fluorescence intensity at the time point t in the bleached cell, I_0 is the relative fluorescence intensity immediately after bleaching, I is the relative fluorescence intensity prior to bleaching and t is the recovery time. A third-party software program, Olympus Fluoview V2.1C (Olympus), was used to automatically analyze the data.

Western blot analysis. Cells were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology), and protein concentrations were quantified by the bicinchoninic acid method (Beyotime Institute of Biotechnology). Equal quantities of total proteins (40 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skimmed milk for 1 h and incubated overnight at 4°C with the following primary anti-

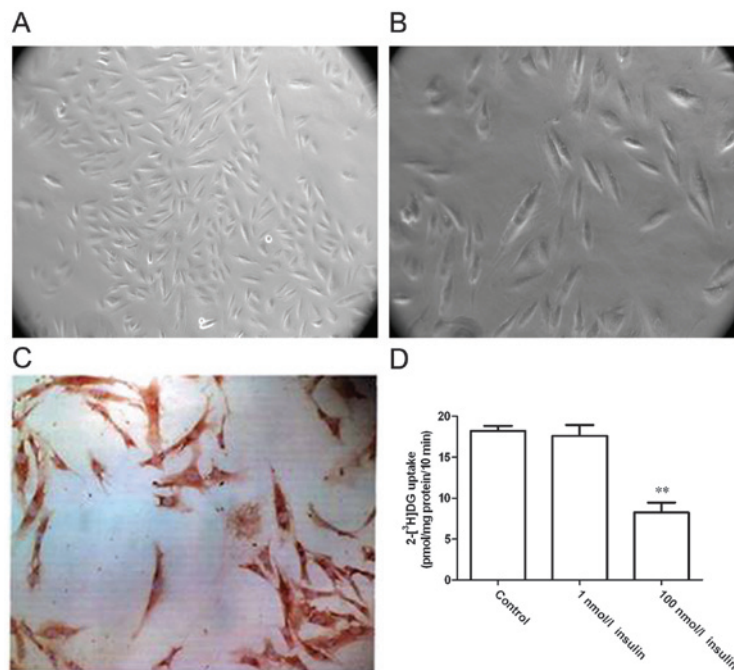


Figure 1. Observations of VSMCs and cell glucose uptake. (A) Primary VSMCs viewed under an inverted microscope (magnification, x100). (B) Primary VSMCs viewed under an inverted microscope (magnification, x200). (C) Immunostaining for α -actin in VSMCs (magnification, x100). (D) Cellular glucose uptake rates, n=5. **P<0.01, compared with the control group. VSMCs, vascular smooth muscle cells.

bodies: Rabbit polyclonal anti-Cx43 (cat. no. ab11370) or rabbit polyclonal anti-p-Cx43 (cat. no. ab30559; Abcam, Cambridge, MA, USA) at dilutions of 1:1,000 and 1:800, respectively. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2054; Santa Cruz Biotechnology, Inc.) at 1:5,000 dilutions for 1 h at 37°C. Subsequently, the blotted protein bands were exposed to and visualized with enhanced chemiluminescence reagents (Millipore). Developed films were digitized by scanning, and the optical densities were analyzed with Image J version 1.43b software (National Institutes of Health, Bethesda, MD, USA). β -actin was used as an internal control for the analysis of protein expression.

Detection of H_2O_2 activity. Intracellular hydrogen peroxide levels were measured using an Amplex Red Hydrogen Peroxide Assay kit (Invitrogen Life Technologies), strictly following the instructions provided in the user manual.

Statistical analysis. All data are presented as the mean \pm standard deviation. Comparisons between groups were conducted using one-way analysis of variance, and P<0.05 was considered to indicate a statistically significant difference. GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used for graph processing.

Results

Identification of VSMCs and the effect on glucose uptake following different doses of insulin. A tissue adherent cultivation approach was adopted to separate VSMCs. After 5-7 days, smooth muscle cells grew out from the edges of the tissue blocks. The cells were fusiform- or spindle-shaped, rela-

tively small in size with ovoid-shaped nuclei, and the cytoplasm displayed strong light refraction (Fig. 1A and B). After culturing for 3-4 weeks, the cells formed monolayers and displayed a typical 'peak-to-valley' growth. Immunocytochemical staining for α -actin, a specific marker of VSMCs, demonstrated that the majority of cells were α -actin-positive with a large quantity of brown-yellowish myofilaments in an arrangement parallel to the longitudinal axis of the cell (Fig. 1C). These observations indicated the successful acquisition of VSMCs. Then, the cells were treated with different concentrations of insulin and glucose uptake was assessed after 24 h (Fig. 1D). Compared with the control group, the glucose uptake rate did not show significant changes in cells treated with 1 nmol/l insulin (P>0.05), although this rate was significantly decreased in cells treated with 100 nmol/l insulin (P<0.01). These results indicate that high-dose insulin reduced the sensitivity of VSMCs to insulin and led to a state of insulin resistance.

High-dose insulin suppresses GJIC and induces the phosphorylation of Cx43 at s368. FRAP assays were performed to evaluate the effect of insulin on GJIC in VSMCs (Fig. 2A and B). The results showed that 1 nmol/l insulin did not induce significant changes in the maximum fluorescence recovery proportion or the fluorescence recovery rate of treated cells, while treatment with 100 nmol/l insulin for 24 h significantly decreased these readouts (P<0.01). These results suggested that high-dose insulin could suppress the GJIC function of VSMCs. Furthermore, western blotting showed that cells treated with 100 nmol/l insulin demonstrated a significantly higher level of phosphorylated Cx43 at s368 and a significantly decreased level of Cx43 (both P<0.05) (Fig. 2C and D). This result indicated that high-dose insulin promotes the phosphorylation of Cx43 at s368 and downregulates the level of Cx43.

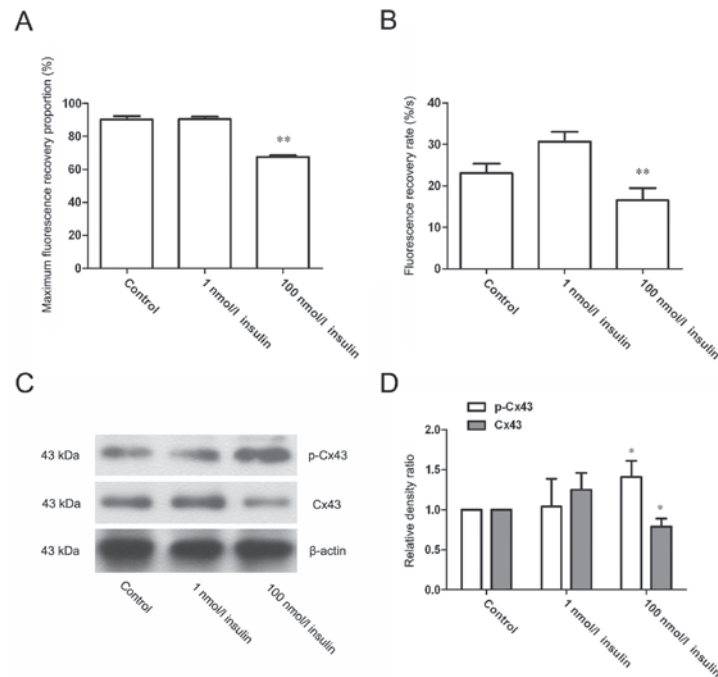


Figure 2. Fluorescence recovery and Cx43 expression in VSMCs treated with different concentrations of insulin. (A) Maximum fluorescence recovery proportion, $n=6$. (B) The fluorescence recovery rate, $n=6$. (C) Western blotting for Cx43 phosphorylation at s368 and Cx43 expression; representative results are presented in the figure. (D) The phosphorylation of Cx43 at s368 and the relative expression level of Cx43, $n=3$. * $P<0.05$ and ** $P<0.01$, compared with the control group. VSMCs, vascular smooth muscle cells; Cx43, connexin 43.

High-dose insulin induces H_2O_2 release from VSMCs. H_2O_2 activity was measured to explore the mechanisms underlying the effect of insulin on GJIC (Fig. 3). Treatment with 1 nmol/l insulin did not exert a significant impact on cellular H_2O_2 activity, while treatment with 100 nmol/l insulin for 24 h significantly enhanced the cellular H_2O_2 level ($P<0.01$), indicating that the effect of high-dose insulin on GJIC may be correlated with the release of H_2O_2 .

High-dose insulin promotes the phosphorylation of Cx43 and inhibits GJIC via the release of H_2O_2 . VSMCs were pre-treated with 2,000 U/ml catalase 30 min prior to the insulin treatment, and then the GJIC level was measured (Fig. 4A and B). Compared with cells treated with only high-dose insulin, the maximum fluorescence recovery proportion and the fluorescence recovery rate were significantly increased in cells pretreated with catalase ($P<0.01$). Western blotting further revealed that compared with cells treated with only high-dose insulin, catalase-pretreated cells showed a significantly decreased phosphorylation level of Cx43 at s368 and an elevated Cx43 expression level (Fig. 4C and D; $P<0.05$).

Discussion

It has been shown that insulin affects the proliferation, migration, phenotypic transformation and cellular contraction of VSMCs, and is involved in the occurrence and development of cardiovascular disease (28-30). GJIC is an important pathway contributing to intercellular substance and signal transmission and thus is crucial in the maintenance of normal vascular function. However, the effect of insulin on intracellular GJIC remains unclear. In this study, using different concentrations of

insulin to treat *in vitro*-cultured VSMCs, it was observed that high-dose insulin suppressed cellular GJIC function, promoted the phosphorylation of Cx43 at s368 and downregulated the expression of Cx43. In addition, high-dose insulin treatment enhanced H_2O_2 release. Furthermore, catalase pretreatment significantly restored GJIC function, which was accompanied by decreased phosphorylation of Cx43 at s368 and increased Cx43 expression. These results indicate that high-dose insulin can regulate GJIC in VSMCs through the mediation of oxidative stress.

Insulin can promote glucose uptake and utilization in tissues and cells, and thus serves as an essential hormone in glucose metabolism. In the clinic, insulin resistance refers to the physiological condition in which reduced glucose uptake and utilization efficiencies lead to a compensatory increase in insulin secretion to maintain glucose hemostasis in the body (31). Hyperinsulinemia is often used as one of the diagnostic criteria for insulin resistance. Due to the limitations of human research, the majority of studies have treated cells with high-dose insulin for studies of the pathogenesis of insulin resistance (32,33). In this study, insulin doses of 1 and 100 nmol/l in VSMCs were used to evaluate its effects on vascular states under physiological and pathological conditions. The results demonstrated that insulin at a physiological concentration did not significantly affect glucose uptake, while high-dose insulin significantly reduced the glucose uptake rate. The findings are consistent with the conclusions drawn in previous studies (34).

Vascular tissue displays abundant GJIC, which is extensively involved in various physiological functions of blood vessels and is closely associated with the occurrence and development of various diseases (35). By treating VSMCs with different concentrations of insulin, it was observed that high-dose insulin could

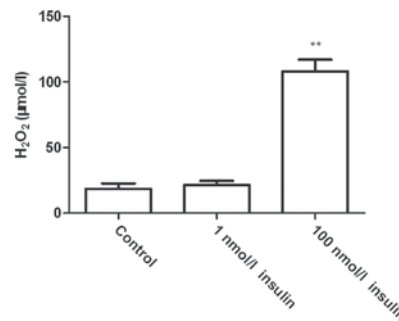


Figure 3. Cellular H₂O₂ content in VSMCs treated with difference concentrations of insulin, n=3. **P<0.01, compared with the control group. VSMCs, vascular smooth muscle cells.

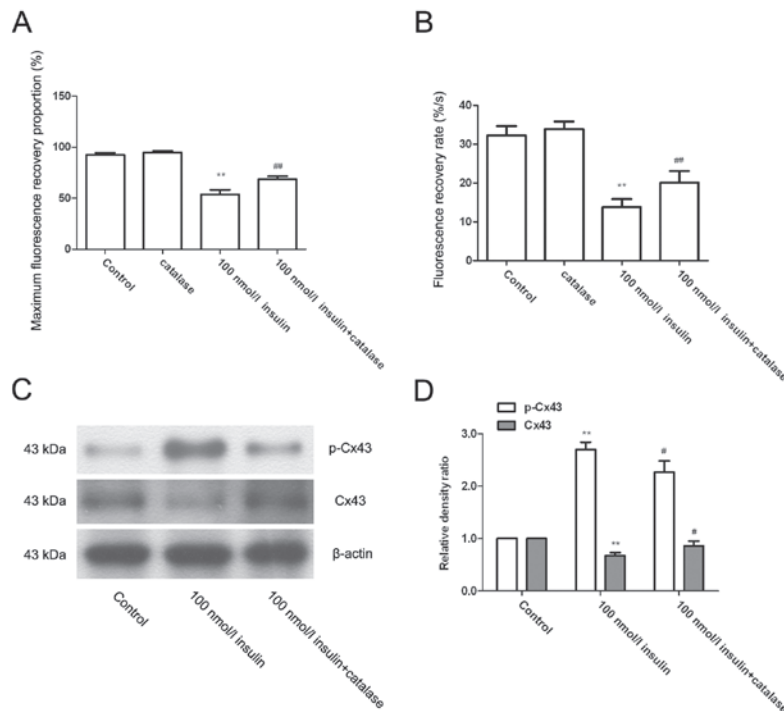


Figure 4. Fluorescence recovery and Cx43 expression in VSMCs following treatment with catalase. (A) The maximum fluorescence recovery proportion, n=6. (B) The fluorescence recovery rate, n=6. (C) Western blotting for Cx43 phosphorylation at s368 and Cx43 expression; representative results from three repeated experiments are presented in the figure. (D) The phosphorylation of Cx43 at s368 and the relative expression level of Cx43, n=3. **P<0.01, compared with the control group. #P<0.05 and ##P<0.01 compared with the 100 nmol/l insulin treatment group. VSMCs, vascular smooth muscle cells.

suppress GJIC in VSMCs. Therefore, abnormal GJIC function may contribute to the pathogenesis of insulin resistance. Among all connexins present in vascular tissue, Cx43 shows the highest protein expression level, and multiple studies have shown that abnormal phosphorylation of Cx43 at s368 can downregulate Cx43 expression and inhibit GJIC function (36,37). In this study, it was observed that high-dose insulin induced abnormal phosphorylation of Cx43 at s368 and downregulated Cx43 expression, indicating that the high-dose insulin-induced reduction of GJIC function may be associated with the abnormal phosphorylation of Cx43 at s368.

Currently, it is hypothesized that high-dose insulin induces insulin resistance, type 2 diabetes (38), atherosclerosis (39), hypertension (40) and obesity (41) primarily through the induction of oxidative stress responses (42,43). H₂O₂ is a stable component of reactive oxygen species in VSMCs and is also a second messenger for various stimulators in smooth muscle

cells (44). The current study demonstrated that high-dose insulin could induce a release of H₂O₂, which is consistent with the conclusions drawn from previous studies (45). In addition, the inhibition of GJIC function by H₂O₂ through the induction of Cx43 phosphorylation has been verified in various types of cells (46,47). In this study, catalase pretreatment reversed the high-dose insulin-induced inhibition of GJIC, decreased the phosphorylation level of Cx43 at s368, and upregulated Cx43 expression. These findings further confirmed that high-dose insulin could promote Cx43 phosphorylation through the induction of H₂O₂ release, thus suppressing GJIC function.

In conclusion, through the activation of oxidative stress responses, high-dose insulin treatment induced Cx43 phosphorylation and downregulated Cx43 expression, thus suppressing GJIC function. This process may contribute to the pathogenesis of insulin resistance, although the detailed mechanisms require further investigation in in-depth studies.

Acknowledgements

This study was supported by a grant from the National Basic Research Program of China (973 Program, no. 2012CB518606).

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