Activation of Wnt signaling reduces high-glucose mediated damages on skin fibroblast cells

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ABSTRACT
Objectives: High-glucose (HG) stress, a mimic of diabetes mellitus (DM) in culture cells, alters expression of a large number of genes including Wnt and NF-κB signaling-related genes; however, the role of Wnt signaling during HG-mediated fibroblast damage and the relationship between Wnt and NF-κB signaling have not been understood. In this study, we aimed to investigate the effects of Wnt signaling on HG-mediated damages.

Materials and Methods: Wnt3a was treated to HG-stressed human primary foreskin fibroblasts and the levels of Wnt signaling markers and cell proliferation were monitored. In addition, Wnt3a and NF-κB signaling inhibitor were assisted to analyze the relationship between two pathways.

Results: The results indicated that HG treatment repressed β-catenin level, and Wnt3a treatment increased the levels of β-catenin and FZD8 as well as cell proliferation. RNA-seq based transcriptome analysis identified 207 up-regulated and 200 down-regulated genes upon Wnt5a supply. These altered genes are distributed into 20 different pathways. In addition, gene ontology (GO) analysis indicates that 20 GO terms are enriched. Wnt signaling genes were further verified by qRT-PCR and the results were similar with RNA-seq assay. Since NF-κB signaling negatively regulates Wnt marker gene expression, Bay117082, a typical NF-κB signaling inhibitor and Wnt3a were supplemented for testing β-catenin and phosphorylated IκBα (p-IκBα), respectively.

Conclusion: HG positively inhibits Wnt signaling and signaling activation via supplementation of Wnt3a rescued the defect caused by HG. NF-κB signaling negatively regulates accumulation of β-catenin, but Wnt signaling has no effects on IκBα activation.

Introduction

Diabetes mellitus (DM), defined as high blood sugar concentration, is the severe metabolic diseases, and its associated complications affect a large number of people in the developed world. The typical symptom of DM is unifit hyperglycemia, which causes severe diabetic complications including heart, kidney, and skin damages. A large number of patients suffering from diabetic complications showed difficulties in skin wound healing (1). High-glucose (HG; hyperglycemia) induces defects in angiogenesis. Blood resupply in the damaged tissues is important for recovery of skin ulcer in patients with DM (2).

Skin ulcer repair needs demand of recruitment of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages and platelets. Fibroblast is one of the important cell layers whose proliferation and migration, collagen deposition and remodeling, wound contraction, and angiogenesis are the necessary process for wound repair. Extracellular matrix (ECM) forms the largest component of the dermal skin layer (3). Fibroblast is mainly participated in producing and remodeling the ECM, and it is also important for the formation of granulation tissue and subsequent skin repair (4, 5). Reactive oxygen species (ROS) are known as a secondary messenger, and its level inside the cell regulates cell proliferation, maturation and differentiation, the key steps of wound healing (6). Fibroblasts in patient with diabetic ulcer are usually large and widely spread in in vitro culture condition compared with the normal fibroblasts in age-matched controls. They often show abnormal endoplasmic reticulum, increased number of vesicular bodies and lost microtubular structure. Therefore, DM affects protein turnover, autonomous trafficking and normal protein secretion in diabetic ulcer fibroblasts (7, 8). Fibroblasts from diabetic ulcer induce defect in cell proliferation that may result in a decrease of ECM protein production and further delayed wound healing (7). HG-induced fibroblast migration was identified as a result of

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RNA deep sequencing

Total RNA extracted from human foreskin fibroblasts cultured in 5.5 mM concentration of glucose containing medium with or without Wnt3a (100 ng/ml, 1 hr) for the RNA-seq experiments. RNA-Seq experiments and data analysis were performed by the Novel Bioinformatics Co., Ltd. (Shanghai, China, http://www.novelbio.com/). The RNA-seq data is deposited in a personal hard disk and available to share upon requested.

Analysis of the pathway and GO category

Differentially expressed genes were identified from statistical analysis testing for association with biological process gene ontology (GO) terms (17). Fisher's exact test was used to classify the GO category, and the false discovery rate (FDR) was used to calculate and correct the P-value (18). Enrichment of GO members among differentially expressed gene sets was found using the one-tailed Fisher's exact test for 2 × 2 contingency tables (19), and it provides a measure of the significance of the function that as the enrichment increases, the corresponding function is more specific and helpful for finding GOs with a more concrete function description in the experiment.

Pathway analysis was utilized to identify the significantly changed pathway of the differential genes according to KEGG, BioCarta, and Reatome databases. Fisher's exact test has been followed by Benjamini-Hochberg (BH) multiple testing correction to select the significant pathway, and the significance between groups was defined by P-value and FDR (20).

Total RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from the fibroblasts of stimulated or un-stimulated with high concentration of glucose (30 mM) and Wnt3a (100 ng/ml, Abcam, Cat no. ab23327, USA). The cell monolayer was rinsed with ice cold phosphate buffered saline (PBS) once. Then, the cells were directly lysed in a culture dish by addition of 1 ml of Trizol Reagent (Trizol Reagent, Invitrogen) per each 3.5 cm diameter dish, and then 0.2 ml of chloroform was added per 1 ml of Trizol Reagent. 2 µg of total RNA was reverse-transcribed to cDNA by using a GoScript Reverse Transcription Kit (Reverse Transcription System, Promega) following the manufacturer's instructions. The transcript levels were normalized against that of GAPDH. Gene specific primer sequences used for qRT-PCR are listed in Table 1.

Western blot analysis

Total proteins were separated on a SDS-PAGE gel after extraction and transferred onto Immobilon-P Transfer Membranes (Millipore, Tokyo, Japan). The membranes were incubated in 1x Tris buffered saline (TBS) containing 5% skim milk and 0.05% Tween-20 for 1-2 hrs and reacted with corresponding primary
antibodies at 4 °C overnight. An anti-β-catenin antibody (1:2000, Abcam, Cat no. ab16051, Cambridge, USA), anti-p-IκBα antibody (1:2000, Abcam, Cat no. 39A1431, Cambridge, USA), anti-IκBα antibody (1:2000, Abcam, Cat no. ab7217, Cambridge, USA), and anti-GAPDH antibody (1:2000, Abcam, Cat no. abcam 9484, Cambridge, USA) were used as primary antibodies. The membranes were incubated for 1 hr with an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (1:2000, Cell Signaling Technology, Inc., Cat. no. 7074, Danvers, MA, USA).

Statistical analysis

Statistical analysis was performed with Prism 5 software package (GraphPad, San Diego, CA). Significant differences were expressed as mean±SE. Comparison between two groups was analyzed by student’s t test, while comparisons between more than two groups were performed by using one-way ANOVA.

Results

HG reduces β-catenin accumulation in skin fibroblasts

Since HG stress changed gene expressions of a large number of genes including Wnt signaling genes (10); therefore, protein level of β-catenin, a key regulator of Wnt signaling, was further analyzed. The results showed that β-catenin level was significantly reduced after 1 hr of HG (30 mM) treatment (Figures 1A, 1B). To test the activation of Wnt signaling by exogenous supply of a recombinant Wnt3a, the fibroblast cells were treated with Wnt3a (100 ng/ml) for 1 and 3 hrs, and β-catenin levels were examined in non-treated (Con) Wnt3a treated cells. The results of Western blot analysis showed that Wnt3a increased the level of β-catenin in 1 and 3 hrs of treatment (Figures 1C, 1D). Also, Wnt3a together with HG treatment for 1 hr inhibited HG effects on β-catenin repression in fibroblasts (Figures 1E, 1F).

Wnt3a dependent transcriptome changes in fibroblasts

To identify Wnt-regulated genes and pathways, RNA-Seq experiments were performed using normal and Wnt3a-treated human fibroblast cells. Since FZD8 and β-catenin levels were obviously changed after 1 hr of treatment (Figure 1B), the fibroblast cells stimulated for 1 hr with Wnt3a and non-treated cells were collected for RNA-Seq analysis. The RNA-Seq results showed that 407 genes were differentially expressed (>1.5 fold change; P-value<0.05) in the Wnt3a-treated fibroblasts. Among them, 200 genes were down-regulated, while 207 genes were up-regulated. GO analysis indicated that 20 GO terms were (P-value<0.01), stress, and ATP activity (Table 2). Further pathway analysis that was performed with

Table 1. qRT-PCR primer sequences

| Primer | Sequences |
|--------|-----------|
| Wnt2 F | AAGCTCATTTGGGGGACCTGG |
| Wnt2 R | CCGGAATGATAGCTGACT |
| Wnt3 F | ATCATAAGGGGGCTGCTGCGAGCTGG |
| Wnt3 R | CTTGAGGTCGACGTCGTAGA |
| Wnt11 F | GAATTGGTCTGACTATGAGT |
| Wnt11 R | GTATTGTCGCTTGAGTCAG |
| TCF7 F | CTGGAGACCCCTGACTTCTT |
| TCF7 R | ATCCCTATGCTGGTGTCCG |
| FZD8 F | CTGGGAGATCCAGTCTC |
| FZD8 R |TTTATGTCATGCAGAGGT |
| GAPDH F | GACCTGGGCTCTGAATAAAC |
| GAPDH R | CTTAGCAATTTGCTGTC |

Figure 1. High-glucose (HG) and Wnt3a effects on β-catenin levels in fibroblast cells. (A) Western blot analysis detected β-catenin levels in normal and HG-treated cells (3 hrs). (B) The band density shown in (A) was measured. The experiments repeated three times. (C) β-catenin levels in normal and Wnt3a (100 ng/ml)-treated cells (1 and 3 hrs after treatment) were analyzed. (D) The band density shown in (C) was measured. The experiments repeated three times. (E) β-catenin levels in the cells with HG or HG together with Wnt3a treatment for 3 hrs were analyzed. (F) The band density shown in (E) was measured. The experiments repeated three times. Significant differences at P-value<0.05 level are indicated by different letters

Wnt3a treatment activated expression of FZD8 and fibroblast cell proliferation

In previous transcriptome study, HG reduced expression of a Wnt-related gene FZD8 and cell proliferation (10). Therefore, FZD8 gene expression and cell proliferation were analyzed after treatment of the cells with HG, Wnt3a and HG together with Wnt3a. The results of qRT-PCR showed that HG and Wnt3a treatment for 1 hr repressed and induced FZD8 gene expression, respectively, while treatment of HG together with Wnt3a exhibited no significant differences from non-treated cells (Con) (Figure 2A). Findings showed that 50 mM glucose obviously inhibited fibroblast cell proliferation (10), but Wnt3a activated cell proliferation of normal and glucose treated (50 mM) skin fibroblast cells (Figure 2B).
Table 2. Gene ontology (GO) classification

| GOID         | GOTerm                                         | Log2 fold | Expression |
|--------------|------------------------------------------------|-----------|------------|
| GO:0042310   | Vasoconstriction                              | 1.81      | up         |
| GO:0006950   | Response to stress                            | 1.47      | up         |
| GO:001480    | Response to gamma radiation                   | 1.54      | up         |
| GO:0071277   | Response to calcium ion                       | -1.34     | down       |
| GO:0033120   | Regulation of RNA splicing                    | -1.45     | down       |
| GO:0019065   | Receptor-mediated endocytosis of virus by host cell | 1.24    | up         |
| GO:1903598   | Positive regulation of gap junction assembly  | 1.81      | up         |
| GO:0001960   | Negative regulation of cytokine-mediated signaling pathway | 1.81 | up         |
| GO:0032400   | Melanosomal localization                      | 1.57      | up         |
| GO:0043409   | MAPK cascade                                   | 1.81      | up         |
| GO:0009062   | Fatty acid catabolic process                  | -1.10     | down       |
| GO:0071207   | Histone pre-mRNA stem-loop binding            | 1.08      | up         |
| GO:006970    | Response to osmotic stress                    | -1.18     | down       |
| GO:0051301   | Cell division                                 | 1.25      | up         |
| GO:0054931   | Positive regulation of mitotic cell cycle     | 1.03      | up         |
| GO:0070836   | Caveola assembly                               | 1.81      | up         |
| GO:0006874   | Calcium ion homeostasis                       | 1.81      | up         |
| GO:0002904   | B cell apoptotic process                      | -1.46     | down       |
| GO:0032780   | Negative regulation of ATPase activity        | 2.61      | up         |
| GO:0002079   | Inner acrosomal membrane                      | 2.04      | up         |

Figure 2. High-glucose (HG) and Wnt3a effects on FZD8 gene expression and cell proliferation. (A) qRT-PCR was performed to monitor the expression level of FZD8. GAPDH was used as an internal control. Data represent mean values ± SE of 3 replicates. (B) Cell proliferation before and after the HG and Wnt3a treatment with 10% fetal bovine serum (FBS) in culture medium was measured by CCK-8 assay after a 72-hr of incubation. Significant differences at P-value<0.05 level are indicated by different letters.

Figure 3. Wnt3a-dependent expression of Wnt signaling genes. qRT-PCR was performed to monitor the mRNA levels of Wnt2, Wnt3, Wnt11, and TCF7. GAPDH was used as an internal control. Data represent mean values ± the SE (n=6 replicates; *P-value<0.05, **P-value<0.01 versus the untreated control group). Differentially expressed genes resulted in 20 different enriched pathways, including Wnt, vascular endothelial growth factor (VEGF), and the transforming growth factor beta (TGF-beta) signaling pathways (Table 3). To verify RNA-Seq results, 4 Wnt signaling related genes (Wnt3, Wnt3, Wnt11, and TCF7) were further monitored by qRT-PCR. Wnt2, Wnt3, and Wnt11 were suppressed, while TCF7 was induced by Wnt3a treatment, and the qRT-PCR results were similar with RNA-seq data (Figure 3).
Table 3. Pathway classification

| Pathway ID   | Pathway Term                                                                 | Log2 fold | Expression |
|--------------|------------------------------------------------------------------------------|-----------|------------|
| PATH:04310   | Wnt signaling pathway                                                        | 1.39      | up         |
| PATH:04370   | VEGF signaling pathway                                                        | -1.34     | down       |
| PATH:04120   | Ubiquitin mediated proteolysis                                               | 1.58      | up         |
| PATH:04350   | TGF-beta signaling pathway                                                   | 1.40      | up         |
| PATH:05222   | Small cell lung cancer                                                       | -1.64     | down       |
| PATH:00400   | Phenylalanine, tyrosine and tryptophan biosynthesis                          | -1.14     | down       |
| PATH:00512   | O-Glycan biosynthesis                                                        | 1.14      | up         |
| PATH:05410   | Hypertrophic cardiomyopathy (HCM)                                            | -1.85     | down       |
| PATH:03440   | Homologous recombination                                                     | 1.36      | up         |
| PATH:05414   | Dilated cardiomyopathy                                                       | -1.85     | down       |
| PATH:00471   | D-GLutamine and D-glutamate metabolism                                       | 1.19      | up         |
| PATH:04610   | Cytokine-cytokine receptor interaction                                       | -1.51     | down       |
| PATH:04710   | Circadian rhythm mammal                                                      | -1.39     | down       |
| PATH:04110   | Cell cycle                                                                   | -1.99     | down       |
| PATH:04260   | Cardiac muscle contraction                                                   | -1.85     | down       |
| PATH:05219   | Bladder cancer                                                               | 1.03      | up         |
| PATH:04360   | Axon guidance                                                                | 1.24      | up         |
| PATH:05412   | Arrhythmogenic right ventricular cardiomyopathy (ARVC)                       | -1.85     | down       |
| PATH:00250   | Alanine, aspartate and glutamate metabolism                                  | -2.24     | down       |

Figure 4. Relationship between NF-κB and Wnt signaling pathways in skin fibroblasts. (A) β-catenin levels were analyzed after Bay117082 (Bay), a typical NF-κB signaling inhibitor, and Wnt3a treatment. (B) The band density shown in (A) was measured. The experiments repeated three times. (C) Western blot analysis was performed to analyze the levels of phosphorylated IκBα and total IκBα (t-IκBα) after Wnt3a treatment for 1 hr. (D) The band density shown in (C) was measured. The experiments repeated three times. Significant differences at P-value<0.05 level are indicated by different letters.

Relationship between NF-κB and Wnt signaling in skin fibroblast cell damage

HG activated inflammatory response including NF-κB and TNF-α signaling. In addition, the NF-κB pathway inhibitor, Bay117082, supply suppressed HG-mediated repression of FZD8, Wnt signaling marker gene (10). Therefore, connection between NF-κB and Wnt signaling was further analyzed. Immunoblotting results indicated that Bay117082 treatment increased the level of β-catenin, slightly higher than Wnt3a (Figures 4A, 4B). In an opposite way, the effect of Wnt3a on IκBα, the most characterized and studied NF-κB regulator, activity was examined. The results of Western blot analysis indicated that Wnt3a treatment did not change both phosphorylated IκBα (p-IκBα) and total IκBα (t-IκBα) levels (Figures 4C, 4D). Taken together, these results suggest that the NF-κB is upstream of β-catenin, the key regulator of Wnt signaling, but Wnt signaling activation via Wnt3a did not change NF-κB activity.

Discussion

Diabetes-induced skin ulcer is hard to be recovered, and is considered as a global health issue in the patients suffering from DM. Skin wound healing process requires coordination of multiple layers of cells including fibroblasts. The fibroblasts are important for synthesizing the ECM and collagen, the structural framework (stroma) for skin tissues. Previous studies on the HG-induced damage of skin fibroblasts identified that abnormal activation of Rac1 and suppression of JNK are tightly associated with skin wound repair (9, 21). Furthermore, transcriptome study regarding HG stress in human skin fibroblast cells identified that many biological processes were altered upon the fibroblasts stressed by HG, including Wnt and inflammatory response...
pathways (10). Here, we observed that HG reduces accumulation of the key Wnt signaling regulator β-catenin, but Wnt3a supply recovered HG effects on expressions of β-catenin and its downstream FZD8 gene (Figures 1B, 1E), suggesting that HG may change β-catenin accumulation to alter Wnt signaling downstream gene expressions. In addition, Wnt signaling activation protected fibroblast cell from HG stress through increased cell proliferation (Figure 2B), implying that Wnt signaling is important for HG-mediated fibroblast damage.

To further understand Wnt signaling activation by Wnt3a, RNA-Seq assay was utilized to analyze. The results determined a large population of differentially expressed genes after Wnt3a stimulation. Among them, 200 genes were down-regulated, while 207 genes were up-regulated. Further, analyses of associated pathways using GO and KEGG database revealed various biological processes and pathways (Table 2 and 3). Compared with the transcriptome data about HG-mediated gene expressions in skin fibroblasts, Wnt3a stimulation changed a largely different group of genes, but Wnt signaling was observed in both analyses (10) (Table 2 and 3). FZD8 gene expression after HG and Wnt3a stimuli showed that HG represses, while Wnt3a induces FZD8 gene expression (Figure 2A), indicating that HG and Wnt3a in opposite way regulate Wnt signaling gene.

Furthermore, inhibition of NF-κB pathway through the treatment of Bay117082 induced β-catenin, but Wnt3a supply did not change IκBα activity, the key component of NF-κB signaling, exhibiting the maintenance of total and phosphorylated IκBα levels (Figure 4). These data suggest that at least IKK/NF-κB signaling is the upstream of β-catenin and Wnt3a treatment did not make influence on IκBα activity. In addition, Wnt3a-induced transcriptome did not cover NF-κB and other inflammatory response pathways genes (Table 3). Previously, Bay117082 treatment induced FZD8 gene expression, but it is reversed by application of Wnt signaling inhibitor, IWR (10). These results are somehow similar with the data presented here, and relationship between Wnt and NF-κB signaling needs to be clarified by testing more markers in the further study. In conclusion, this study identified that HG affects human skin fibroblast cells partially via inhibition of Wnt signaling, and inhibition of NF-κB or activation of Wnt signaling by application of inhibitor or ligand molecules reserve the HG-induced damages. The results presented here will be useful for further understanding molecular basis of diabetes-induced skin ulcer.

**Conclusion**

Findings of the present study showed the effects of HG stress in skin fibroblast cells. Treatment of Wnt3a to activate Wnt signaling somehow protects HG-induced damages on fibroblasts. Wnt3a treatment induced a large number of gene alterations and inflammatory response signaling located upstream of Wnt signaling to protect HG damages on skin fibroblast cells.

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