The Role of PPARγ in Hyperglycemia-induced Deleterious Effect on Chondrocytes

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Research article

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Abstract

Aims: There is a well-established link between OA and diabetes, and study have shown that hyperglycemia might play an important role in the occurrence and development of OA. Accumulative evidence suggested that PPARγ was involved in AGEs-related disease, including diabetes and OA. The study was designed to investigate the effects of hyperglycemia on the expression of PPARγ in chondrocytes and whether PPARγ agonist pioglitazone had a chondroprotective effect.

Main methods: Primary human chondrocytes were incubated with different concentration of glucose medium (5.5mM-30mM) in the presence or absence of PPARγ agonist pioglitazone. The AGEs formation level in chondrocytes culture medium was detected by AGEs specific ELISA kits. The expression of IL-1, MMP-13, TNF-α, PPARγ was determined by western blotting and real-time PCR.

Key findings: The AGEs formation level was time-dependently and dose-dependently increased in chondrocyte culture media. Hyperglycemia could enhance the expression of IL-1β, TNF-α, MMP-13, but the level of PPARγ was decreased in a time-dependent and dose-dependent manner, which was inhibited by PPARγ agonist pioglitazone. Noteworthy, the maximum effect was found to at 20mM glucose medium for 24h.

Significance: Hyperglycemia could increase the AGEs formation level and induce inflammatory response and matrix degradation reaction in chondrocytes. PPARγ agonists pioglitazone had a chondroprotective effect via inhibit inflammatory response and matrix degradation reaction. PPARγ could be a potential target for pharmacologic intervention in the treatment of diabetic-induced OA.

Introduction

Osteoarthritis (OA) is one of the most common arthritis condition, characterized by progressive degeneration and destruction of articular cartilage, mainly manifested as joint pain, deformity and dysfunction [1]. OA is a serious disease with increasing impact worldwide [2], that affected over 300 million individuals globally in 2017, and it may become the 4th leading cause of disability worldwide by 2020 [3]. Traditionally, it was believed that OA was mainly caused by mechanical factors and age-related cartilage degeneration. However, There is a well-established link between OA and metabolic syndrome [4–8]. Waine H et al first described the correlation between diabetes and OA in 1961 [9]. Moreover, Berenbaum F first proposed the concept of diabetes-induced OA in 2011, and suggested that hyperglycemia might play an important role in the occurrence and development of OA [10]. Among adults with diabetes, the prevalence of doctor-diagnosed arthritis were nearly half (47.1%) [11]. In a population-based cohort study, Schett G et al showed that severe clinical symptoms of OA and structural joint changes was observed in OA patients with type 2 diabetes after adjustment for age, BMI, and other risk factors for OA [12].

Prolonged hyperglycemia induces oxidative stress, overproduction of pro-inflammatory and advanced glycation end products (AGEs) in cartilage as well as in the vessels, heart, kidneys, eyes, nerves. AGEs, the
products of spontaneous reaction of reducing sugars with proteins or non-enzymatic glycation, also accumulate in OA cartilage leading to matrix stiffness, resulting in deleterious effects on cartilage matrix\textsuperscript{[13,14]}. Furthermore, our previous study has indicated that AGEs could bind to its receptors RAGE, and plays a pro-catabolic, pro-inflammatory role in the pathogenesis of OA via NF-κB and MAPK pathway in chondrocyte\textsuperscript{[15,16]}.

Peroxisome proliferator-activated receptors gamma (PPAR\textsubscript{γ}) is a member of the ligand-activated nuclear hormone receptor superfamily\textsuperscript{[17]}. PPAR\textsubscript{γ} exhibits originally the function of regulating fatty acid uptake, insulin sensitivity and glucose homeostasis. In addition, recent studies have demonstrated that PPAR\textsubscript{γ} plays a crucial role in AGEs induced chondrocyte damage in vitro and in vivo. Pioglitazone, one of PPAR\textsubscript{γ} agonists, has been confirmed that could reduce the severity of the AGEs-induced OA in a rabbit model\textsuperscript{[18]}. In human chondrocytes incubated with AGEs, the expression of IL-1, TNF-α, and MMP-13 was up-regulate, but the level of PPAR\textsubscript{γ} was decreased, which was inhibited by PPAR\textsubscript{γ} agonist and suggested that PPAR\textsubscript{γ} could be a potential target for pharmacologic intervention in the treatment of OA\textsuperscript{[15]}. However, the role of PPAR\textsubscript{γ} in hyperglycemia-induced chondrocyte deleterious effect is still unclear. In this study, we put forward the hypothesis that PPAR\textsubscript{γ} down-regulation in chondrocyte induced by hyperglycemia might be responsible for hyperglycemia-induced production of TNF-α, IL-1β and MMP-13.

Materials And Methods

Reagents and Antibodies

IL-1β, PPAR\textsubscript{γ}, MMP-13 antibody was purchased from WUHAN SANYING (Hubei Province, China). TNF-α antibody were purchased from Abcam (USA). Pioglitazone were purchased from MedChemExptess (U.S.A). Penicillin/streptomycin solution, fetal bovine serum (FBS), low-glucose Dulbecco's modified Eagle's medium (DMEM), type II collagenase, and trypsin were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture and treatments

Human primary chondrocytes were purchased from Chi Scientific Inc. (Catalog No. 1–0002, Jiangyin, Jiangsu, China). All experiments were conducted using chondrocytes within 1–4 passages. Chondrocytes was cultured in complete DMEM supplemented with 15% fetal bovine serum(FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. Chondrocytes were exposed from normal glucose concentration(5.5 mM) to high glucose concentration(10–30 mM) for 3, 6, 12 or 24h\textsuperscript{[19]}. In order to investigate the role of PPAR\textsubscript{γ} in the hyperglycemia-induced deleterious effect on chondrocytes, chondrocytes (> 85% confluent) were incubated in high glucose medium in the presence or absence of PPAR\textsubscript{γ} agonist pioglitazone(50 µM).

Western Blotting

The cellular lysates were prepared. Equal protein (40 µg) was seperated on a 10% SDS gel and transferred into an immobilon-P (PVDF) membrane (Millipore). Western blotting was performed as described
previously\textsuperscript{[19]}. Densitometric analysis of the scanned bands was performed using Image J (MD, USA) according to the manufacturer’s instructions.

**Enzyme Linked Immunosorbent Assay (ELISA)**

After treatment, the supernatant of culture medium was collected. The AGE formation level in the supernatants was quantified by specific AGEs kits according to the manufacturer’s instruction (CloudClone, Wuhan, China). The absorbance was read immediately at 450 nm.

**Total RNA Extraction and Real-time Fluorescent Quantitative PCR**

Total RNA was extracted from chondrocytes using TRizol (Invitrogen, USA) according to the protocol of the kit. 1 µg total RNA was reverse transcribed to synthesize cDNA according to the reverse transcription kit (VAZYME, Nanjing, China). cDNA was amplified with real-time PCR on ABI7900 real-time PCR detection system using QuantiFast SYBR Green PCR Kit (VAZYME, Nanjing, China) according to the manufacturer’s instructions. Real-time PCR was performed using the specific primers: β-actin forward 5’-AGCGAGCATCCCCCAAGTT-3’ and reverse 5’-GGGCACGAAGGCTCATCATT-3’, TNF-α forward 5’-TCAGAGGCCCTGACCTCAT-3’ and reverse 5’-GGAAGACCCCCTCCAGATAG-3’, IL-1β forward 5’-TCCAGCTACGAATCTCCGAC-3’ and reverse 5’-TGATCGTACAGGTGCATCGT-3’, MMP13 forward 5’-CCACAACCTAAACATCCAA-3’ reverse 5’-AAACAGCTCCGATCAACC-3’, PPARγ forward 5’-GGTTGTCGCATCTTTTCACT-3’ reverse 5’-GCTACCAGCATCCGTCTTT-3’. The relative mRNA expression abundance was normalized to the housekeeping gene β-actin using the formula $Y = 2^{-\Delta\Delta Ct}$.

**Statistical Analysis**

The data represent the mean ± SD of three independent experiments. The significance of differences was evaluated by using one-way analysis of variance(ANOVA) followed by Student’s t-test to compare means. The difference is significant when $P < 0.05$. SigmaPlot 12.0 and SPSS 13.0 software were engaged in data analyzing and plotting.

**Results**

AGEs accumulates in chondrocyte culture media treated with hyperglycemia

In diabetic patients, hyperglycemia could increase the concentration of AGEs formation\textsuperscript{[20, 21]}. To evaluate the concentration of AGEs in chondrocyte culture fluid treated with hyperglycemia, we incubated the chondrocytes with different glucose concentrations (5.5 mM, 10 mM, 20 mM, 30 mM), and collected chondrocyte culture media at 3 h, 6 h, 12 h, and 24 h, respectively. The AGEs level was detected by AGEs specific ELISA kits. As shown in Fig. 1, the AGEs level was time-dependently and dose-dependently increased in chondrocyte culture media. To note, the maximum effect was found to at 20 mM glucose medium for 24 h.
Effect of hyperglycemia to the inflammatory response and matrix degradation reaction in chondrocyte

Chondrocytes were incubated with glucose medium (5.5 mM, 10 mM, 20 mM, 30 mM) for 3–24 h. The mRNA expression levels of MMP-13, IL-1β and TNF-α increased in a time-dependent and dose-dependent way (Fig. 2). The protein expression of MMP-13, IL-1β and TNF-α were dose-dependently increased in chondrocytes treated with high glucose medium both for 3 h, 6 h, 12 h, 24 h (Fig. 3). However, there was no significant difference between the two groups of 30 mM and 20 mM groups ($P > 0.05$). Noteworthy, when the chondrocytes was treated with 20 mM glucose medium, the expression of MMP-13, IL-1β and TNF-α was up-regulated in a time-dependent way (Fig. 4).

Hyperglycemia down-regulated the expression of PPARγ in chondrocyte

Human articular chondrocytes were cultured into 6-well plates, and were incubated with chondrocytes with different glucose media (5.5 mM, 10 mM, 20 mM, 30 mM) for 3 h, 6 h, 12 h, 24 h. The mRNA and protein of PPARγ expression was detected by Real Time-PCR and western blotting. Hyperglycemia can induce the decrease of PPARγ mRNA expression in a concentration- and time-dependent manner, and the maximum effect is 20 mM for 24 h (Fig. 5A). The protein expression of PPARγ was increased dose-dependently in chondrocytes treated with high glucose medium both for 3 h (Fig. 5B), 6 h (Fig. 5C), 12 h (Fig. 5D), and 24 h (Fig. 5E). However, there was no significant difference between the two groups of 30 mM and 20 mM groups ($P > 0.05$). Noteworthy, the maximum effect was found to at 20 mM glucose medium for 24 h (Fig. 5F). Therefore, subsequent experiments were performed with 20 mM glucose medium and 24 h.

The Effect of pioglitazone on hyperglycemia-induced inflammatory response and matrix degradation reaction in chondrocyte

Our previous study had shown that PPARγ agonist pioglitazone could inhibit the effect of AGEs-induced inflammatory response and matrix degradation reaction both in vivo and in vitro [15, 16, 18, 22, 23]. In order to further explore the protective effect of PPARγ in chondrocytes treated by hyperglycemia, chondrocytes was incubated with high glucose medium (20 mM) supplemented with pioglitazone (50 mM) for 24 hours. As shown in Fig. 6 and Fig. 7, pioglitazone could reverse hyperglycemia-induced down-regulation of PPARγ, and reverse the the effect hyperglycemia-induced up-regulaton of MMP13, IL-1β, TNF-α. These results indicated that inflammatory response and matrix degradation reaction in chondrocyte induced by hyperglycemia could be inhibited by PPARγ agonist pioglitazone.

**Discussion**

Articular cartilage is a non-innervated and non-vascularized tissue, and it is composed of extracellular matrix and chondrocytes. Chondrocytes are the only cells in articular cartilage, responsible for synthesis of the extracellular matrix. The imbalance of anabolic and catabolic activities in chondrocytes is the leading cause of OA. Glucose is essential for articular chondrocytes to maintain homeostasis, generate energy, and synthesize extracellular matrix. Chondrocytes which are glycolytic cells can express glucose
transporters (GLUT) and sense the glucose concentration in the media such as extracellular matrix, synovial fluid, and subchondral bone\textsuperscript{[24]}. Chondrocytes can adapt themselves to extracellular glucose level, but these adaption do not work during OA. Chronic hyperglycemia in cartilage which induces oxidative stress, overproduction of pro-inflammatory and pro-degradative cytokines and AGEs, which may modulate OA progression \textsuperscript{[6,25]}. In diabetic patients, hyperglycemia could increase the concentration of AGEs formation\textsuperscript{[20,21]}. Meanwhile, AGEs also accumulate with age in OA cartilage modifying its mechanical properties, including stiffness and resistance. AGEs also can bind to its receptor RAGE inducing a pro-inflammatory and pro-catabolic effect on chondrocytes\textsuperscript{[15,16,18,22,23]}. In streptozotocin-induced diabetic rat models, the formation of AGEs in cartilage is significantly increased, and the expression of secreted pro-degradative cytokines such as MMP-13 and inflammatory factors such as IL-1 and TNF-\(\alpha\) is significantly increased, but type II collagen reduced\textsuperscript{[26]}. H. Liang \textit{et al} had shown that the treatment of high concentrations of glucose (15–45 mM) for 72 hrs, did not affect the cell viability of chondrocytes\textsuperscript{[27]}. In our study, the concentration of glucose were set from 5.5 mM(control) to 30 mM. Our results showed that local high glucose in chondrocyte culture media can increase the AGEs formation in time-dependently and dose-dependently way. Moreover, the expression of MMP-13, IL-1\(\beta\), TNF-\(\alpha\) increased in a time-dependently and dose-dependently way treated with high-glucose medium, the maximum effect was 20 mM glucose medium for 24 h. These results indicated that hyperglycemia may play an important role in modulating anabolic and catabolic activities in chondrocytes.

The peroxisome proliferator-activated receptors (PPAR) belong to the nuclear hormone receptor superfamily and have a diverse role in a wide ranged of tissues, including the regulation of glucose and lipid metabolism, inflammation, and diabetes \textsuperscript{[28,29]}. Studies have demonstrated that PPAR\(\gamma\) agonist pioglitazone could reduce the severity of OA in animal models \textsuperscript{[30,31]}. Cartilage-specific PPAR\(\gamma\) knockout mice exhibit the spontaneous osteoarthritis phenotype, which further provide evidence that PPAR\(\gamma\) is a critical regulator of cartilage health \textsuperscript{[32]}. Accumulative evidence suggested that PPAR\(\gamma\) was involved in AGES-related disease, including diabetes and diabetes-related complications\textsuperscript{[33–38]}, cardiovascular disease\textsuperscript{[39]}, non-alcoholic fatty liver disease (NAFLD)\textsuperscript{[40]}, cognitive dysfunction and dementia\textsuperscript{[41]}, and OA\textsuperscript{[22,42]}. Based on the common points of PPAR\(\gamma\) in diabetic and OA physiopathologic mechanism, we speculate that PPAR\(\gamma\) can inhibit the deleterious effects of high glucose on chondrocytes.

Pioglitazone is an anti-diabetic drug to improve insulin resistance and pleiotropic effects on insulin secretion. Our previous study had shown that PPAR\(\gamma\) agonist pioglitazone could inhibit the effect of AGES-induced inflammatory response and matrix degradation reaction both in vivo and in vitro\textsuperscript{[15,16,18,22,23]}. In the present study was designed to investigate the effects of hyperglycemia on the expression of PPAR\(\gamma\) in chondrocytes and whether PPAR\(\gamma\) agonist pioglitazone had a chondroprotective effect. In this context, our data have shown that the PPAR\(\gamma\) expression was down-regulated in chondrocytes treated by high glucose in time-dependently and dose-dependently way. Pioglitazone could reverse hyperglycemia-induced down-regulation of PPAR\(\gamma\), and reverse the the effect hyperglycemia-induced up-regulation of MMP13, IL-1\(\beta\) and TNF-\(\alpha\).
Conclusions

In conclusion, our study demonstrated that hyperglycemia could increase the AGEs formation level and induce inflammatory response and matrix degradation reaction in chondrocyte. PPARγ agonists pioglitazone had a chondroprotective effect via inhibit inflammatory response and matrix degradation reaction. We suggested that hyperglycaemia induced deleterious effect on chondrocytes via increasing the AGEs formation and decrease the expression of PPARγ. PPARγ agonists pioglitazone could be a potential target for pharmacologic intervention in the treatment of diabetic-induced OA.

Declarations

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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AGEs accumulates time-dependently and dose-dependently in chondrocyte culture media treated with hyperglycemia. Primary human chondrocytes were incubated with different glucose concentrations (5.5-30mM) for 3-24h. The level of AGEs formation in the supernatants was detected by specific ELISA kits. All data are expressed as means ± SD and are representative of three independent experiments. * P< 0.05 versus 3h group, & P <0.05 versus 5.5mM group, # P> 0.05, versus 20mM control.
Figure 2

Induction of IL-1β, TNF-α and MMP-13 mRNA expression by hyperglycemia in human chondrocytes. The mRNA expression of IL-1β, TNF-α and MMP-13 was quantified by real-time PCR. All data are expressed as means ± SD and are representative of three independent experiments. * P< 0.05 versus 3h group, # P> 0.05, versus 20mM control.
Figure 3

Induction of IL-1β, TNF-α and MMP-13 protein expression by hyperglycemia in human chondrocytes for 3h(A-C), 6h(D-F), 12 h(G-I), 24 h(J-L). The protein expression of IL-1β, TNF-α and MMP-13 was quantified by western blotting using β-actin as an internal control. Densitometric analysis for IL-1β, TNF-α and MMP-13 levels corrected to β-actin is shown. All data are expressed as means ± SD and are representative of three independent experiments. * P< 0.05 versus 5.5mM group, # P> 0.05, versus 20mM control.
Figure 4

Induction of IL-1β, TNF-α and MMP-13 protein expression by 20mM glucose medium in human chondrocytes for 3-24h. The protein expression of IL-1β, TNF-α and MMP-13 was quantified by western blotting using β-actin as an internal control. Densitometric analysis for IL-1β, TNF-α and MMP-13 levels corrected to β-actin is shown. All data are expressed as means ± SD and are representative of three independent experiments. * P< 0.05 versus 3h group.
Figure 5

The effect of hyperglycemia on PPARγ expression in human chondrocytes. A The chondrocytes were incubated with different glucose medium (5.5mM-30mM), the PPARγ mRNA expression was detected by real-time PCR. All data are expressed as means ± SD and are representative of three independent experiments. * P< 0.05 versus 5.5mM 3h group, # P> 0.05, versus 20mM 24hcontrol. In B-E The chondrocytes were incubated with different glucose medium (5.5mM-30mM) for different time (3h(B), 6h(C), 12h(D), 24h(E)), western blotting was used to detect the protein level of PPARγ expression. * P< 0.05 versus 5.5mM group, # P> 0.05, versus 20mM control. F The chondrocytes was incubated with 20mM glucose medium for 3-24h, western blotting was used to detect the protein level of PPARγ expression. * P< 0.05 versus 3hgroup. B-F Densitometric analysis for levels corrected to β-actin is shown. All data are expressed as means ± SD and are representative of three independent experiments.
Figure 6

Effects of pioglitazone on hyperglycemia-induced MMP-13, IL-1β, TNF-α and PPARγ mRNA expression. The mRNA expression levels of MMP13 (A), IL-1β (B), TNF-α (C) and PPARγ were detected by qPCR. * P<0.05 versus HGM (20mM) group.
Figure 7

Effects of pioglitazone on hyperglycemia-induced MMP-13, IL-1β, TNF-α and PPARγ protein expression. The protein expression levels of MMP13 (A), IL-1β (B), TNF-α (C) and PPARγ were detected by western blotting. Densitometric analysis for levels corrected to β-actin is shown. All data are expressed as means ± SD and are representative of three independent experiments. * P< 0.05 versus HGM (20mM) group.