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Altered expression of base excision repair genes in response to high glucose-induced oxidative stress in HepG2 hepatocytes

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Jing Pang[1ABCDEFG], Chao Xi[2BCEFG], Yang Dai[3BC], Huan Gong[1BC], Tie-mei Zhang[1ADEFG]

[1] The Key Laboratory of Geriatrics, Beijing Hospital & Beijing Institute of Geriatrics, Ministry of Health, China
[2] College of Life Sciences, Beijing Normal University, China
[3] Graduate School of Peking Union Medical College and Chinese Academy of Medical Sciences, China

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Summary

Background: It is widely accepted that chronic hyperglycemia induces DNA oxidative damage in type 2 diabetes, but little is known about the effect of hyperglycemia on the DNA repair system which plays a critical role in the maintenance of genomic DNA stability in diabetes.

Material/Methods: To investigate the alteration of base excision repair (BER) genes under hyperglycemia, the relative expression of the mRNAs of the BER genes – *ogg1, polβ, lig3, xrcc1,* and *parp1* – were quantified using real-time PCR in HepG2 hepatocytes incubated with 5.5 mM or 30 mM glucose.

Results: High levels of glucose induced ROS accumulation and DNA damage, paralleling the dynamic alterations of BER mRNA expression. Compared to 5.5 mM glucose-treated cells, *ogg1* and *pol β* mRNA expression transiently increased at day 1 and decreased after day 4 in cells exposed to 30 mM glucose. Exposure to 30 mM glucose increased the activity of PARP1, which led to reduced cellular NAD content and insulin receptor phosphorylation.

Conclusions: Exposure to high concentrations of glucose initially led to the increased expression of BER mRNAs to counteract hyperglycemia-induced DNA damage; however, long-term exposure to high glucose concentrations reduced the expression of mRNA from BER genes, leading to accumulated DNA damage.

key words: hyperglycemia • base excision repair genes • PARP1 activation

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Author's address: Tie-mei Zhang, Beijing Institute of Geriatrics, Beijing Hospital, No. 1 Dahualu, Beijing, 100730, China, e-mail: tmzhang126@126.com

Background

Persistent hyperglycemia plays a major role in β-cell dysfunction and insulin resistance, which leads to the further progression of diabetes [1]. One potential central mechanism for hyperglycemia is the formation of excess reactive oxygen species (ROS) [2,3]. ROS accumulation leads to oxidative DNA damage, which is detected by several sensor proteins that activate DNA repair pathways [4,5]. Imperfections in DNA repair may induce changes in the genetic information, leading to cell death or disease, which implies that cell fate can be determined by the ability to repair DNA.

Diabetes-associated DNA damage has been widely described [6,7], and DNA repair mechanisms are initiated to counteract hyperglycemia-induced oxidative DNA damage [8]. Base excision repair (BER) is the major pathway involved in the repair of hyperglycemia-induced oxidative base modifications, which is different from the radiation-induced DNA repair system [9,10], depending on a series of enzymatic reactions. Several BER enzymes have been found to be related with diabetes. In the first step of the BER pathway, 8-oxoguanine DNA glycosylase (OGG1) recognizes and excises 8-hydroxylation of guanine bases (8-oxodG) [11]. The expression of OGG1 is significantly increased in the islets of patients with type 2 diabetes and positively correlates with disease progression [12]. Additionally, the activation of the DNA repair enzyme, poly(ADP-ribose) polymerase 1 (PARP1), is increased in diabetic patients compared to normal controls [13]. Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia. While it is clear that hyperglycemia induces DNA damage via the production of ROS, the effect of hyperglycemia on DNA repair enzymes has not been investigated. We therefore investigated whether the BER enzymes *ogg1, pol β, lig3, xrcc1, and parp1* were involved in glucose toxicity.

As hyperglycemia-induced cellular dysfunction accumulates *in vivo* over a long period of time, the time courses of mRNA expression of BER genes under high glucose treatment were investigated. The altered expression of BER mRNAs in response to high glucose concentration may provide a model with which to further explore the molecular mechanisms of diabetes disease progression.

Material and Methods

Cell culture

Human hepatoma HepG2 cells were obtained from the Concord Cell Center (Peking Union Medical College, China) and were cultured in Eagle's Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA) containing 5.5 mM glucose supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells were cultured in a 5% CO_2-humidified atmosphere at 37°C. For high glucose treatment, the culture media was supplemented with 30 mM glucose.

Measurement of intracellular ROS generation

ROS determination was performed as previously described [14], based on the oxidation of the non-fluorescent DCFH (Beyotime, Shanghai, China) to the fluorescent dye 2',7'-dichlorofluorescein (DCF) by peroxide. The intensity of fluorescence was immediately measured with flow cytometry (BD, Franklin Lakes, NJ) at an excitation of 488 nm and emission of 530 nm.

Table 1. Sequences of primers used for real-time PCR.

Gene name	Forward primer	Reverse primer
Ogg1	TGTCCATATGTGGCACATTGC	AAGTTTCCCAGTTCCTTGTTGG
PoL β	CAACCAAACAGCCAAAACTG	TTACTGGGAAGCTGGCAAAC
Lig3	ACGCTGTGCCAAACAAAGG	CGTCGAATGCCACAAAGTAGC
Parp1	ACACCCCTTGCACGTACTTC	GATGGGTTCTCTGAGCTTCG
Xrcc1	ACGGATGAGAACACGGACAGTG	CGTAAAGAAAGAAGTGCTTGCCC

Comet assay

DNA damage was analyzed by the alkaline single-cell gel electrophoresis comet assay, as previously described [15]. The slides were observed under a microscope and the tail moment and olive tail moment were calculated from 100 randomly selected images from each sample using the CASP Comet Assay software.

Quantitative real-time PCR

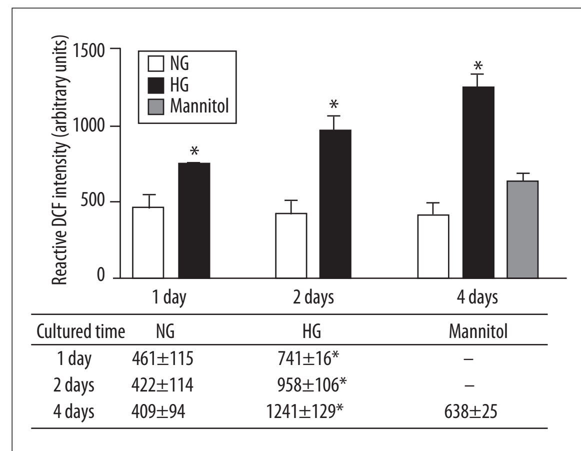
Total RNA was extracted from HepG2 cells using Trizol (Invitrogen), and cDNA was synthesized from 2 µg total RNA using M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA). Real-time PCR was performed in a Lightcycler (Applied Biosystems, Foster City, CA) using the SYBR Green Master Mix Kit. PCR primers were constructed for *ogg1,* DNA polymerase beta *(pol β),* ligase III (*lig3),* X-ray repair cross complementing group 1 (*xrcc1)* and *parp1*, based on the published nucleotide sequences of human gene regions. A list of the PCR primers is shown in Table 1. *Hprt* served as an internal control, and the expression of the transcripts was quantified as a ratio of *Hprt* expression.

Estimation of NAD levels

Intracellular NAD content was measured using the NAD+/NADH quantification kit (k337; BioVision, Mountain View, CA) according to the manufacturer's instructions.

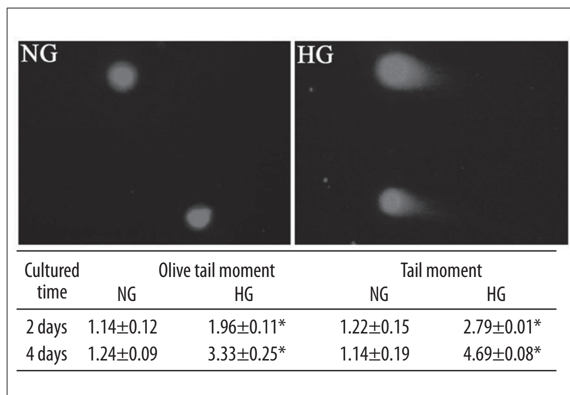
Western blot analysis

Samples (50 µg protein) were separated by 9% SDS-PAGE, transferred to PVDF membrane and blocked with 5% milk in Tris-buffered saline with Tween (TBST). Samples were then incubated overnight at 4°C with antibodies for anti-poly(ADP-ribose) (PAR) (Trevigen, Gaithersburg, MD), anti-PARP1, anti-insulin receptor β (InsR β) or anti-phospho-InsR β (p-Tyr1150/1151-InsR) (Cell Signaling Technology, Danvers, MA). Bound antibodies were detected with HRP-linked secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized using enhanced chemiluminescence. To determine if InsR was phosphorylated, we stimulated cells with 100 nM insulin for 20 min before lysis.



Cultured time	NG	HG	Mannitol
1 day	461±115	741±16*	–
2 days	422±114	958±106*	–
4 days	409±94	1241±129*	638±25

Figure 1. ROS generation induced by treatment with different concentrations of glucose. ROS production was estimated fluorometrically by flow cytometry and was expressed as DCF fluorescent intensity. The following abbreviations were used throughout the figures: NG, normal glucose (5.5 mM glucose); HG, high glucose (30 mM glucose). Bars represent the mean ± SD (n=3). * P<0.05 *vs.* NG.



Cultured time	Olive tail moment NG	Olive tail moment HG	Tail moment NG	Tail moment HG
2 days	1.14±0.12	1.96±0.11*	1.22±0.15	2.79±0.01*
4 days	1.24±0.09	3.33±0.25*	1.14±0.19	4.69±0.08*

Figure 2. DNA damage induced by treatment with different concentrations of glucose. Comet tail was observed under fluorescence microscope and DNA damage assessed by the olive tail moment and tail moment using comet assay software. The values represent the mean ±SD (n=3). * P<0.05 *vs.* NG.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Comparisons between 2 means were performed using the independent-samples t-test; multiple comparisons amongst groups were analyzed using one-way ANOVA with SPSS software 11.0. Significance was defined as $p<0.05$.

Results

High glucose concentration increases ROS generation and DNA damage

No significant differences in ROS accumulation were observed in cells cultured with 5.5 mM glucose for 1, 2 or 4 days (Figure 1) or 24.5 mM-mannitol (permeability control). Extended exposure of HepG2 cells to 30 mM glucose induced ROS accumulation and DNA damage. Compared to 5.5 mM glucose-treated cells (control), 30 mM glucose led to a 3-fold increase in ROS accumulation at day 4 (Figure 1, $p<0.05$). Additionally, the extent of DNA damage, indicated by the olive tail moment and tail moment, was increased in cells treated with 30 mM glucose compared to cells treated with 5.5 mM glucose (Figure 2, $p<0.05$).

High glucose concentration induces transcription of BER mRNAs

The mRNA expression of BER proteins was examined in HepG2 cells cultured in 5.5 or 30 mM glucose for 1 to 4 days to assess how the DNA repair pathway responded to DNA damage during long-term high glucose treatment.

The expression of the mRNAs of BER genes did not significantly change in HepG2 cells cultured with 5.5 mM glucose at any time-point. In cells treated with high levels of glucose, the expression of *ogg1* increased 1.8-fold at day 1, which then decreased after day 2, compared to cells cultured in 5.5 mM glucose ($p<0.05$, Figure 3A). In cells treated with 30 mM glucose, the expression of *pol β* increased at day 1 (2.7-fold) and day 2 (3.2-fold) and decreased after day 4, compared to cells treated with 5.5 mM glucose ($p<0.05$, Figure 3B). *Lig3* mRNA expression increased over time in 30 mM glucose-treated cells, with a 2-fold increase observed day 4, compared to cells treated with 5.5 mM glucose ($p<0.05$, Figure 3C).

The mRNA expression levels of both *parp1* and *xrcc1* were higher in cells treated with 30 mM glucose compared to cells treated with 5.5 mM glucose from day 1 to day 4; however, these differences were not significant (Figure 3D, E).

High glucose concentration reduces insulin receptor phosphorylation accompanied by PARP1 activation and cellular NAD depletion

Since we have previously demonstrated that PARP1 activation modulates insulin sensitivity through NAD depletion [14], we examined the dynamic expression of PARP1 activity, intracellular NAD content and insulin receptor phosphorylation in high glucose concentrations. PARP1 protein expression was not significantly affected by high glucose (Figure 4A). We determined the total poly(ADP-ribosyl)ated protein content by Western blotting as a measure of PARP1 activity, and observed that PARP1 activity in 30 mM glucose-treated cells was approximately 2.2-fold higher than in 5.5 mM glucose-treated cells after day 6 ($p<0.05$, Figure 4B). Additionally, 30 mM glucose reduced the intracellular NAD content to approximately 50% of the NAD content in 5.5 mM glucose-treated cells at day 4 ($p<0.05$, Figure 4C). At day 2, 30 mM glucose had no significant effect on cellular NAD content and insulin receptor phosphorylation in response to insulin (Figure 4). However, insulin receptor phosphorylation was markedly attenuated after 4 days of treatment with 30 mM glucose ($p<0.05$, Figure 4D), which suggests that glucose toxicity accumulates gradually.

Discussion

The accumulation of oxidative damage as a result of chronic hyperglycemia is a causal link to tissue dysfunction in diabetes

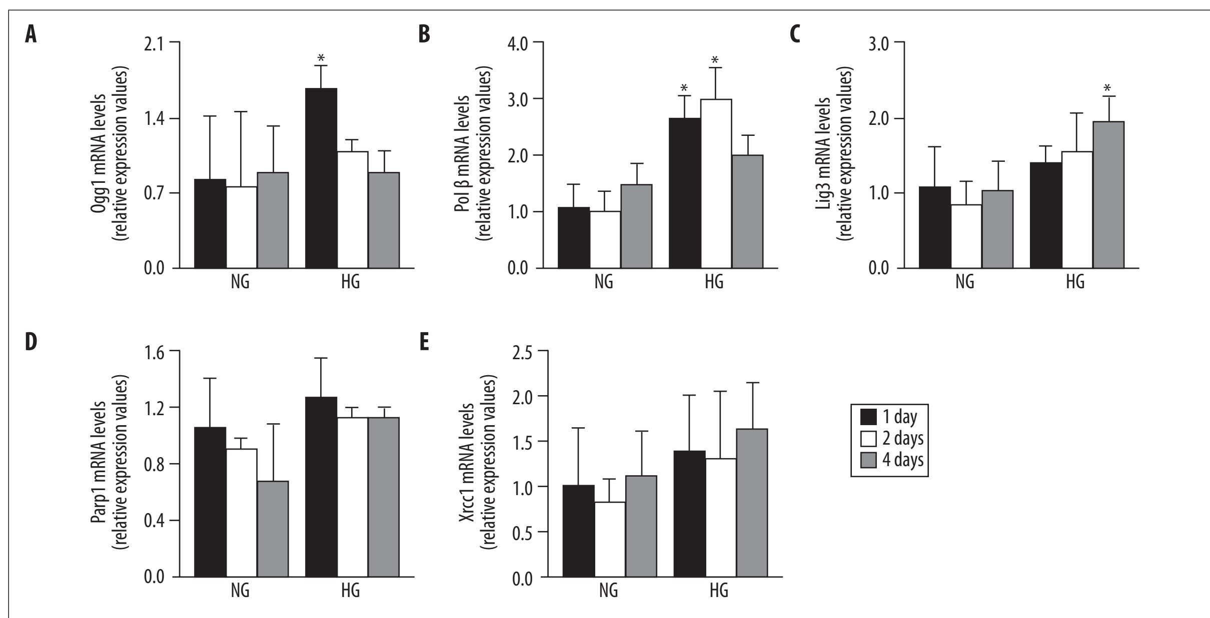


Figure 3. The relative expression of base excision repair genes induced by treatment with different concentrations of glucose. HepG2 cells were cultured in 5.5 mM glucose (NG) and 30 mM glucose (HG) for 1, 2 and 4 days. The mRNA levels of *ogg1* (**A**), *pol β* (**B**), *lig3* (**C**), *parp1* (**D**) and *xrcc1* (**E**) were examined using real-time quantitative RT-PCR. Bars represent the mean ±SD (n=3). *P<0.05 *vs.* NG.

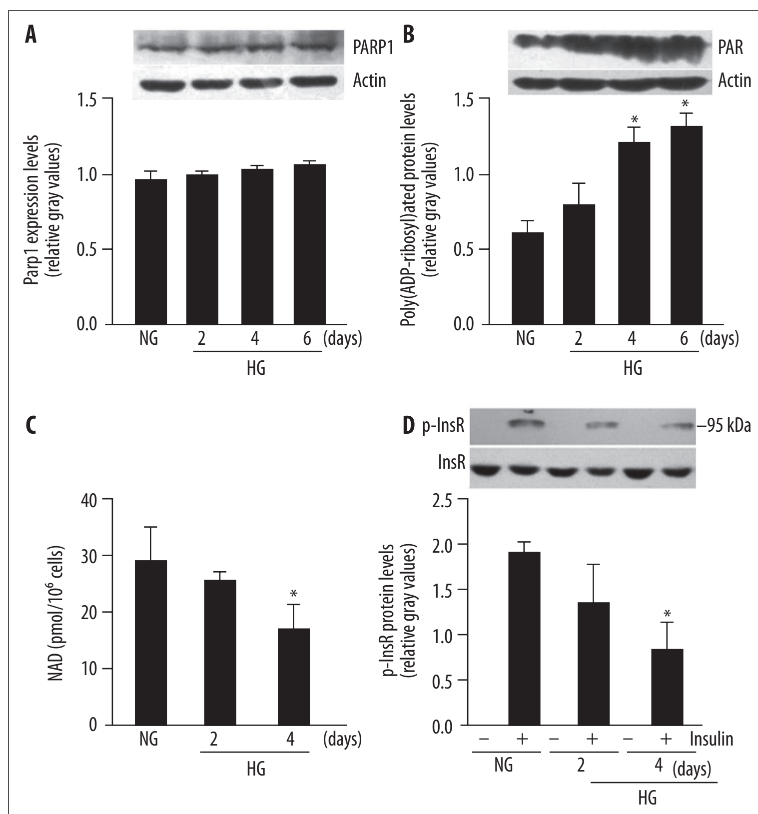


Figure 4. High glucose concentration induced NAD content and insulin sensitivity gradually decreased accompanied by Parp1 activation. Parp1 protein level was not significantly affected by high glucose (**A**). But the activity of Parp1 was induced by 30 mM glucose (**B**), while NAD levels (**C**) and insulin receptor phosphorylation (**D**) both decreased in a time-dependent manner. Actin and insulin receptor (InsR) were used as control to confirm equal loading of proteins. Bars represent the mean ±SD (n=3). * P<0.05 *vs.* NG

[16,17]. DNA oxidative damage can induce transcription and translation errors, which possibly contributed to pancreatic β-cell dysfunction and insulin resistance. Fortunately, the DNA repair system repairs the damaged DNA and maintains normal functions in cells. In this study we demonstrated that genes in the base excision repair pathway changed significantly in response to high glucose treatment, which contributed to altered DNA repair ability and genome stability.

ROS generation is a basic event in oxidative damage, so in the first part of this study we demonstrated that glucose intake caused a significant increase in ROS generation,

which was consistent with a previous study by Mohanty [2]. However, in diabetes there are abnormalities of lipid metabolism and an increase in lipolysis, free fatty acid (FFA) concentrations and triglycerides. Several studies have demonstrated that an increase in FFA concentrations induces ROS generation, lipid peroxidation and inflammation [18,19]. Therefore, the oxidatively-mediated damage may be from a combination of hyperglycemia and elevated FFA concentrations. The source of ROS generation is an exciting focus in diabetes research, and deserves further investigation.

An increase in *ogg 1* and *pol* β mRNA levels was observed in HepG2 cells after 1-day culture in a high glucose environment; this temporal increase may counteract DNA damage induced by oxidative stress. This finding was consistent with the results of another report, in which *pol* β expression was induced by oxidizing agents [8]. However, after long-term exposure to high glucose, both of them reduced to the level of the normal glucose group. Our experiments also revealed that DNA damage increased gradually with the time of high glucose treatment. High glucose treatment for 4 days caused the predominance of DNA damage over DNA repair defense system, leading to DNA damage accumulation, which may induce genomic instability and cell dysfunction. The molecular mechanism of how high glucose modulates BER enzymes expression needs to be investigated further. Previous studies have demonstrated that high glucose treatment decreases OGG1 expression via a redox-dependent activation of Akt in murine proximal tubular epithelial cells, which provides a mechanism of oxidative stress-mediated DNA damage in diabetes [20]. Simone et al. [20] also detected an initial increase in OGG1 expression, but the high glucose treatment time was short compared to our study, which may have been due to the use of different cell lines.

We observed that the BER accessory protein mRNAs, *parp1* and *xrcc1,* exhibited similar expression patterns in response to high glucose, but neither of them were significantly altered by high glucose treatment in this study. Since PARP1 activation has been proved to be related to diabetic complications [13], we then examined the expression and activity of PARP1. Our results show that treatment with high levels of glucose increased PARP1 activity, but did not significantly alter PARP1 protein expression. This suggests that the modulation of *parp1* expression by high glucose concentration is regulated in a post-translational manner. We have previously demonstrated that PARP1 activation leads to the downregulation of intercellular NAD content and insulin receptor (InsR) phosphorylation in HepG2 cells [14]. Therefore, we also used intracellular NAD content and insulin receptor phosphorylation level to evaluate the extent of high glucose damage. High glucose treatment decreased intercellular NAD and insulin receptor phosphorylation in a time-dependent manner in a hepatoma cell line. These results confirm that long-term high glucose treatment gradually induces the accumulation of cellular dysfunction [21].

Conclusions

The altered expression of BER protein mRNAs in response to high glucose levels suggests functional roles for the BER repair system in genomic stability. In hyperglycemia, the accumulation of DNA damage due to the imbalance between DNA damage and repair may lead to further disease progression towards aggressive diabetes, which should be investigated in future studies. The altered expression of BER mRNAs may have potential as biomarkers to estimate disease progression in patients with diabetes.

Conflict of interest

All authors in this study are free of conflict of interest.

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