Effect of nicotinamide mononucleotide on insulin secretion and gene expressions of *PDX-1* and *FoxO1* in RIN-m5f cells

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Abstract: **Objective** To investigate the effect of nicotinamide mononucleotide (NMN) on insulin secretion and gene expressions of pancreatic and duodenal homeobox 1 (*PDX-1*) and forkhead box-containing protein O-1 (*FoxO1*), which were important transcription factors for insulin secretion. **Methods** Insulin secretion level in RIN-m5f cells was detected by rat insulin ELISA detection kit. The mRNA expression levels of PDX-1 and FoxO1 in RIN-m5f cells were analyzed by real-time PCR. The protein expression of PDX-1 was measured by Western blot. **Results** Insulin secretion levels in RIN-m5f cells treated with repaglinide (10 nmol/L) plus NMN (100 μmol/L) was significantly higher than those in the blank control, the DMSO control group, and the NMN (50 μmol/L) treated group ($P<0.05$). The mRNA expression levels of *PDX-1* in RIN-m5f cells treated with NMN (10, 50 and 100 μmol/L) for 36 h were significantly higher than those in the control group ($P<0.05$, $P<0.01$, and $P<0.001$, respectively). There was marked differences in the mRNA expression levels of *PDX-1* among different concentrations of NMN ($P<0.001$), but no significant differences in the mRNA expression level of *FoxO1* ($P>0.05$). No significant difference was found in the protein expression levels of PDX-1 in RIN-m5f cells treated by NMN (50, 100, and 200 μmol/L) for 36 or 48 h compared with the control group ($P>0.05$). **Conclusion** NMN can stimulate insulin secretion and upregulate the mRNA expression of *PDX-1* in RIN-m5f cells.

Key words: nicotinamide mononucleotide; pancreatic and duodenal homeobox 1; forkhead box-containing protein O-1; RIN-m5f cell

Date of reception 2010-11-05

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Foundation items This work was supported by the National High-Tech R&D Program of China ("863" Program) (2009AA22704), the National Natural Science Foundation of China (30873089, 81173129), the Program for Changjiang Scholars and Innovative Research Team in University (IRT0946), the Open Foundation of Innovative Platform in University of Hunan Province of China (10K078), and the Science and Technology Plan Key Grant of Hunan Province of China (2009TP4068-2), the Fundamental Research Funds for the Central Universities (201023100001).

烟酰胺单核苷酸对 RIN-m5f 细胞中胰岛素分泌及 *PDX-1* 和 *FoxO1* 基因表达的影响

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[摘要] **目的:**在细胞水平研究烟酰胺单核苷酸(nicotinamide mononucleotide, NMN)对胰岛素分泌的调节作用及其对与胰岛素分泌相关的重要转录因子胰十二指肠同源盒基因(pancreatic and duodenal homeobox-1, *PDX-1*)和分叉头框家族转录因子 1(forkhead box-containing protein O-1, *FoxO1*)基因表达的影响。**方法:**采用大鼠胰岛素 ELISA 试剂盒检测 RIN-m5f 细胞胰岛素分泌水平。用 Real-time PCR 检测 RIN-m5f 细胞 *PDX-1* 和 *FoxO1* 的 mRNA 表达水平。用 Western 印迹检测 RIN-m5f 细胞 PDX-1 蛋白表达水平。**结果:**用瑞格列奈10 nmol/L + NMN 100 μmol/L 处理 RIN-m5f 细胞 48 h,与空白对照及 DMSO 对照组相比,胰岛素分泌量均显著增高($P<0.05$);与 NMN 50 μmol/L 组比较,胰岛素分泌量的增高也有统计学意义($P<0.05$)。10,50 和 100 μmol/L 的 NMN 作用 RIN-m5f 细胞 36 h,*PDX-1* 的 mRNA 表达量均上调(依次为 $P<0.05$, $P<0.01$, $P<0.001$)。100 μmol/L 剂量组与 10 μmol/L 和 50 μmol/L 剂量组比较差异也有统计学意义($P<0.001$)。50,100 和 200 μmol/L 的 NMN 作用 RIN-m5f 细胞 36 或 48 h,PDX-1 的蛋白表达量与对照组比较差异无统计学意义($P>0.05$)。**结论:**NMN 可以调控 RIN-m5f 细胞中胰岛素的分泌及 *PDX-1* 的 mRNA 表达水平。

[关键词] 烟酰胺单核苷酸; 胰十二指肠同源盒基因; 分叉头框家族转录因子 1; RIN-m5f
DOI:10.3969/j.issn.1672-7347.2011.10.005

Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme of nicotinamide converting into nicotinamide mononucleotide (NMN). NMN is an important intermediate in the synthesis of nicotinamide adenine dinucleotide (NAD)[1]. Nampt can regulate insulin secretion in pancreatic β cells as a systemic NAD biosynthetic enzyme. A study[2] in white origin population showed that Namp genetic polymorphism might play a role in the development of Type 2 diabetes mellitus (T2DM) possibly by modulating chronic and low-grade inflammatory responses.

Pancreatic and duodenal homeobox-1 (PDX-1) and forkhead box-containing protein O-1 (FoxO1) are 2 important transcription factors related to insulin secretion. PDX-1, also known as steroidogenic factor 1 (STF-1), IDF-1, IDX-1, IPF-1, plays an important role in the regeneration, differentiation, and apoptosis of β cells[3]. Previous study[4] showed that chronic high blood glucose activates oxidative stress and reduces DNA binding activity of PDX-1 and then results in dysfunction of β cells. Decrease of the nuclear localization of PDX-1 could inhibit insulin gene transcription[5]. Some studies[6-8] found that PDX-1 could influence transcription of several other important β cell-specific genes, including glucose transporter 2 (GLUT2)[6], islet amyloid polypeptide[7], and glucokinase[8]. Down-regulation of glucagon-like peptide-1 receptor (GLP-1R) caused by the deficiency of PDX-1 might lead to β cell dysfunction[9]. A Pro63fsdelC deletion mutation in the coding region of PDX-1 protein caused pancreatic agenesis, which is a pathopoiesia gene of adult-typed type 4 diabetes mellitus happened in teenagers (maturity-onset diabetes of the young, MODY4)[10].

FoxO1 is highly expressed in the liver, adipose tissue, and β cells. FoxO1 regulates glucose and lipid formation in the liver. Recent studies suggested that *FoxO1* gene may be involved in insulin/insulin-like growth factor-1 (IGF-1)/insulin receptor substrate-2 (IRS-2) pathway, and it could regulate β cells function, especially glucose-stimulated insulin secretion (GSIS) function[11]. Insulin and IGF-1 cause FoxO1 phosphorylated by PI3K/Akt pathway, which results in FoxO1 moving to the cytoplasm from the nucleus and decreased transcriptional activity of FoxO1[12]. It was reported[13] that PDX-1 was regulated by another transcription factor FoxA2. FoxO1 and FoxA2 have the same DNA binding site in the promoter of PDX-1. FoxO1 inhibited PDX-1 transcription through occupying the binding site of FoxA2. Thus,

FoxO1 may be a negative regulator of PDX-1.

In consideration that Nampt plays an important role in insulin secretion, glucose metabolism, and functional regulation of pancreatic β cells, we aimed to explore the impact of NMN on the cellular level of insulin secretion and the gene expressions of transcription factor *PDX-1* and *FoxO1*.

1 MATERIALS AND METHODS

1.1 Cell culture

The insulin-producing cell line (RINm5F) used in this study was generously gifted from the laboratory of professor Zhou, Department of Endocrinology, the Second Xiangya Hospital of Central South University, Changsha, China. RINm5f cells were subclone of RIN-m cell lines and had a higher rate of insulin secretion. RIN-m5f cells were cultured in RPMI-1640 (Gibco company, USA) containing 10% fetal calf serum (Sijiqing Biological Engineening Materials Co., Ltd., Hangzhou, China) at 37℃ with 5% CO_2.

1.2 Measurement of insulin secretion

Cells were seeded in 6-well microplates at a density of 5×10^5 cells and grown for 48 h in medium with 10 mmol/L glucose. We set up 6 groups to treat with different drugs: 1) the blank group; 2) the group that dimethyl sulfoxide (DMSO) adding to final concentration 2‰; 3) the group treated with 10 nmol/L repaglinide; 4) the group treated with 50 μmol/L NMN; 5) the group treated with 10 nmol/L repaglinide plus 50 μmol/L NMN; 6) the group treated with 10 nmol/L repaglinide plus 100 μmol/L NMN. The medium was collected and centrifuged for 10 min at 5 000 r/min, and the supernatant was frozen at −20 ℃ for insulin measurement. The cells were digested with 0.05% trypsin-0.02% EDTA and counted. Quantitative insulin measurement was conducted using rat insulin ELISA kit (R&D, MN, USA). The insulin concentration was normalized by the cell numbers and expressed as IU per 10^6 cells.

1.3 RNA isolation and semi-quantitative RT-PCR

RNA was extracted from RIN-m5f cells using TRIzol reagent (Invitrogen, CA, USA). Real-time PCR analyses were performed using the SYBR® Premix Ex Taq™ (Perfect Real Time) kit (Takara, Dalian, China) in Mx3000P Real time PCR (Stratagene, DE, USA). Primers used in amplification of *PDX-1*, *FoxO1*, and β-actin were as follows, respectively: sense primer 5′-AAACCGTCGCATGAAG TGGAA-3′, antisense primer 5′-CGAGGTTACGGC ACAATCCTG-3′; sense primer 5′-TACGCCGACCT-CATC-ACCA-3′, antisense primer 5′-GCACACTCT-TCACCATCCACTC- 3′; sense primer 5′-GGAGAT-TACTGCCCTGGCTCCTA-3′, antisense primer 5′-GACTCATCGTA-CTCCTGCTTGCTG-3′. Relative gene expression level was normalised to the internal standard gene β-actin.

1.4 Protein preparation and Western blot

Protein samples from RIN-m5f cells were prepared as previously described[14]. In brief, cells were lysed in TNN-SDS buffer (150 mmol/L NaCl, 50 mmol/l Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 1 mmol/L dithiothreitol, 0.1% SDS, and 2 mmol/L phenylmethylsulfonyl fluoride) at 4 ℃ for 30 min. Lysates were centrifuged at 10 000 r/min for 5 min at 4 ℃ and was stored at −20 ℃. Protein concentration was measured by the use of Bradford method with reagents from Bio-Rad (Beyotime Institute of Biotechnology, Shanghai, China)[15]. Western blot analyses were performed based on described previously with a minor modification[14]. Proteins (50 μg) from each experimental group were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. Membranes were incubated with appropriate dilutions of primary antibodies (PDX-1, 1∶50, Santa Cruz, CA, USA; β-actin, 1∶2 000, Beijing 4A Biotechnology Co., Ltd., Beijing, China), followed by the application of appropriate IRDye™ 800CW marked secondary antibodies (LI-COR, NE, USA). Proteins were detected using Odyssey Near infrared two-color laser imaging system software (LI-COR, NE, USA). Densitometric quantification of bands at subsaturation levels was performed using Gel-Pro analyzer 4 (Media Cybernetics, USA) and normalised to appropriate loading controls, the housekeeping protein β-actin. Data were calculated as the relative abundance of protein levels to the loading control β-actin.

1.5 Statistical analysis

All data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was carried out by SPSS software (version 13.0 for windows, SPSS Inc., Chicago, Illinois, USA). One-way ANOVA was used for multiple comparisons. LSD-*t* test was

used to calculate difference between groups. $P < 0.05$ was considered statistically significant.

2 RESULTS

2.1 Effect of NMN on insulin secretion level

As shown in Fig. 1, the insulin secretion levels in RIN-m5f cells treated with 10 nmol/L repaglinide plus 100 μmol/L NMN were significantly higher than those in the blank group, the DMSO group, and the 50 μmol/L NMN treatment group ($P < 0.05$).

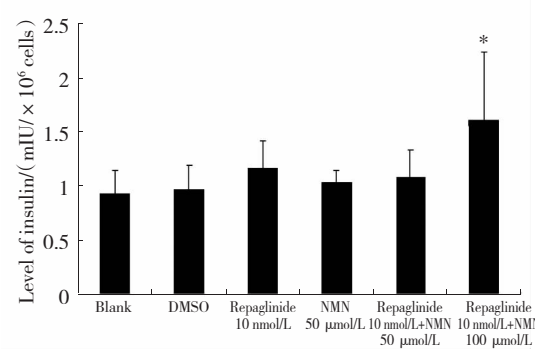

Fig. 1 Effect of NMN on insulin secretion in RIN-m5f cell line. Compared with the blank group and 50 μmol/L NMN treatment group, $*P < 0.05$.

2.2 Influence of NMN on mRNA expressions of *PDX-1* and *FoxO1*

The mRNA expression levels of *PDX-1* in RIN-m5f cells treated by 10, 50, and 100 μmol/L NMN for 36 h were significantly higher than those in the blank group ($P < 0.05$, $P < 0.01$, $P < 0.001$, respectively; Fig. 2). Additionally, there were marked differences in mRNA expression levels of *PDX-1* in RIN-m5f cells treated by 100 μmol/L NMN compared with 10 and 50 μmol/L group ($P < 0.001$). However, there were no significant differences in the mRNA expression levels of *FoxO1* in RIN-m5f cells treated by 10, 50, and 100 μmol/L NMN for 24, 36, and 48 h compared with the blank group ($P > 0.05$, Fig. 3).

2.3 Effect of NMN on protein expression of PDX-1

As shown in Fig. 4, we found that there were no significant differences in the protein expression levels of PDX-1 in RIN-m5f cells treated by 50, 100, and 200 μmol/L NMN for 36 or 48 h compared with the blank group ($P > 0.05$).

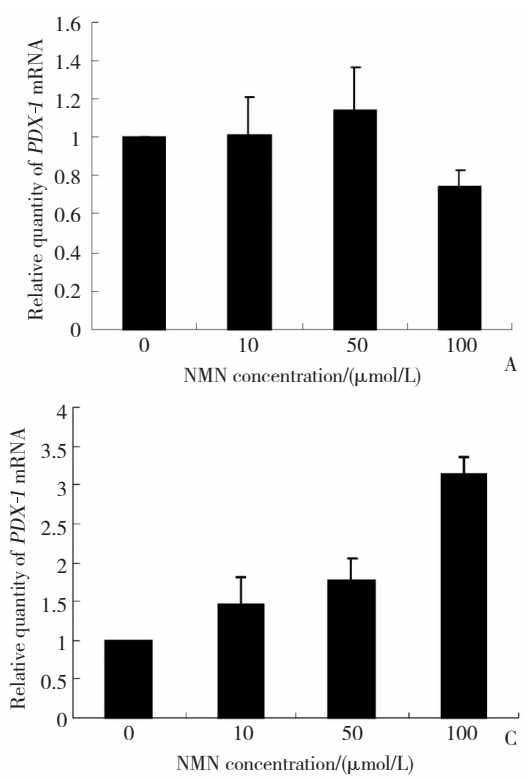

Fig. 2 Effect of NMN on the mRNA expression of *PDX-1* in RIN-m5f cell line. A, B, and C: the mRNA expression levels of *PDX-1* in RIN-m5f cells treated with NMN for 24, 36, and 48 h, respectively. Compared with the control group, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; compared with the groups treated with 10, 50 μmol/L NMN, ### $P < 0.001$.

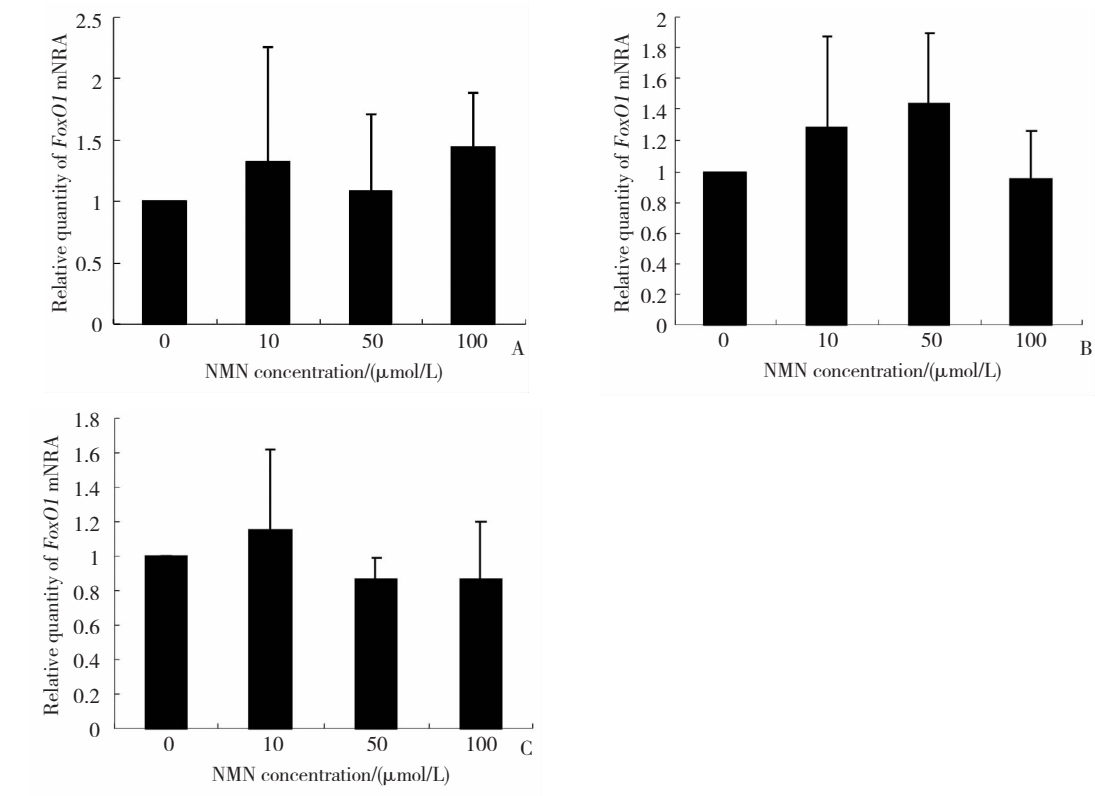

Fig. 3 Effect of NMN on the mRNA expression levels of *FoxO1* in RIN-m5f cell line. A, B, and C: the mRNA expression levels of *FoxO1* in RIN-m5f cells treated with NMN for 24, 36, and 48 h, respectively.

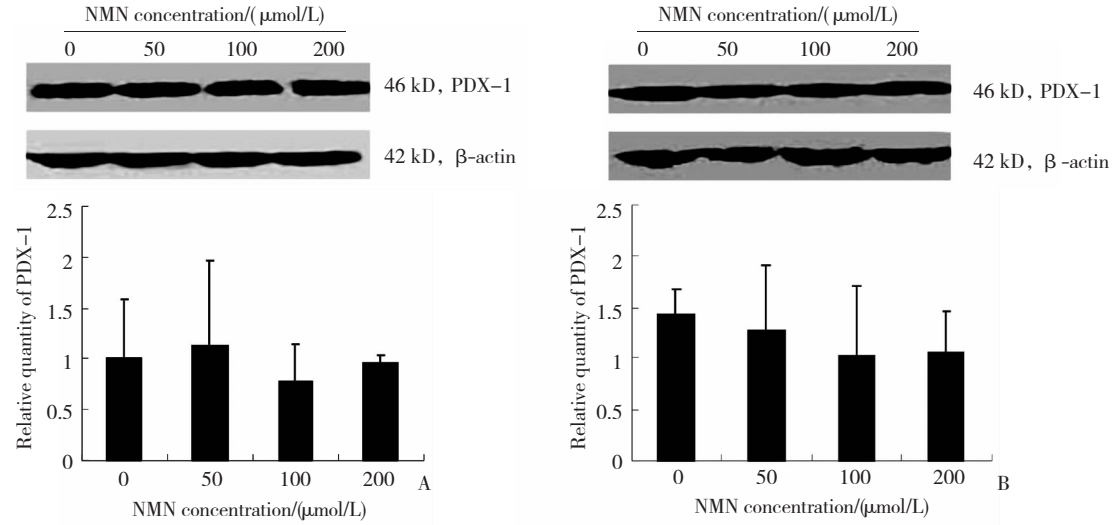

Fig. 4 Effect of NMN on the protein expression levels of PDX-1 in RIN-m5f cell line. A and B: protein expression levels of PDX-1 in RIN-m5f cells treated with NMN for 36 and 48 h, respectively.

3 DISCUSSION

Numerous transcription factors encoding important proteins involved in insulin secretion and glucose metabolism may modify disease susceptibility to diabetes mellitus. Up to date, the pathophysiological mechanism of the occurence and development of diabetes mellitus is still unclear. In the current study, we observed that 10 nmol/L repaglinide plus 100 μmol/L NMN could significantly stimulate insulin secretion in RIN-m5f cells, and also found that NMN increased the mRNA expression levels of transcript factor *PDX-1*, but not *FoxO1*.

It is reported that the high level of NMN in circulation maintained by Nampt played an important role in the normal β cell function[1]. So using NMN to treat β cells could indirectly reflect the influence

of Nampt on β cell function. The mechanism currently known was that NMN stimulated insulin secretion by inhibiting uncoupling protein 2 (UCP 2) expression in β cells through NAD-dependent enzyme Sirt1, increasing ATP levels and promoting glucose-stimulated insulin secretion[16]. NMN increased ATP content in β cells through Sirt1 and closed the K^+-ATP channels of β-cell membrane, which suggested NMN has a synergistic effect on repaglinide. It was consistent with our results in this study.

In the present study, treating RIN-m5f cell line with NMN for a certain time could influence the mRNA expression of *PDX-1*. However, protein expression of PDX-1 in RIN-m5f cell line showed no change after cells were treated with NMN for 36 or 48 h. So the protein expression of PDX-1 may be influenced via many other transcript factors. Recent study has reported that a weak inhibitor of poly ADP-ribose polymerases (PARP) could protect RIN-m5f cells from free fatty acid-induced apoptosis by raising PDX-1 expression[17]. NAD levels could act on NAD- dependent PARP, so NMN may regulate the expression of PDX-1 through PARP pathway. It was recently reported that Nampt over-expression could inhibit FoxO1 expression mediated by SIRT1[18]. However, we did not observe effect of NMN on mRNA or protein expression of FoxO1 in RIN-m5f cell. It may be due to different materials and methods used in different experiments or their complex functions of NMN and FoxO1.

In summary, Nampt is an important rate-limiting enzyme for nicotinamide converting into NMN and plays a very important role in insulin secretion in pancreatic β cells. Intermediate NMN could stimulate insulin secretion and up-regulate the mRNA expression of *PDX-1* in RIN-m5f cells. So we think that Nampt and NMN may influence the susceptibility of diabetes mellitus.

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(Edited by GUO Zheng)