The relationship between isosorbide mononitrate and apoptosis in the rat brain cells with insulin resistance

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Abstract: Objective: To observe the apoptosis-promoting effects and its mechanism of Isosorbide mononitrate on the rat brain cell with insulin resistance. **Methods**: 40 male Wistar (W) rats, 14 week-old, each of them was respectively or jointly fed with normal diet, high fat and high glucose diet（HFHG）, normal saline（NS）and ISMN. They were randomly divided into normal & NS group（Control）, HFHG & NS group（HFHG）, normal & ISMN group（ISMN ） and HFHG & ISMN group（UNITED）, with 10 rats in each group. After 12 weeks, blood samples were taken by carotid artery intubation to assess serum levels of the fasting plasma glucose and insulin. Insulin resistance index (HOMA-IR) was calculated. The brain tissue of each rat was cut to 4 pieces, and TUNEL staining was performed to examine the level of neuronal apoptosis, PCR or Western-blotting was used to determine the levels of NO, iNOS, Bcl-2 mRNA and Bax mRNA including their protein expression. **Results:** 1. The mean levels of NO, iNOS and Bax mRNA including its protein in the groups fed with HFHG and /or ISMN were higher than that of the control group, while bcl-2 mRNA and its protein were lower ($p<0.05$); 2. The NO level of the rat brain tissue was correlated with HOMA-IR in the HFHG group and the HOMA-IR was significantly correlated with NO level in the ISMN group ($p<0.05$). **Conclusions:** 1. ISMN can induce rat brain cell apoptosis of all the groups with treatment of ISMN, and IR aggravates the apoptosis. 2. There is a mutual induction between IR and NO in the process of apoptosis; 3. Protective effect or toxic effect of NO is dependent on the generating ways and the quantity of its.

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Keywords：Isosorbide mononitrate；Nitric oxide synthase；Insulin resistance；Brain cell apoptosis

1. Introduction

The population of metabolic syndrome (MS) including hypertension, insulin resistance (IR), obesity, lipid metabolism disorder, etc. is growing, associated with reduced life span and quality of life. Its prevalence rate is more than 14% in China. MS is a term associated with the collection of a series of risk predictors that are associated with metabolic diseases including cardiovascular diseases, diabetes mellitus (DM) and nervous system disease [1]. Cerebrovascular disease is the leading cause of death in China, and places a huge burden on the individual, family, and community. Several lines of evidence suggest that nerve apoptosis phenomenon exists in cerebrovascular diseases, neurodegenerative disorder, acute brain damage and epilepsy. There are many factors which can cause apoptosis, and studies both in vivo and in vitro are confirmed that excessive NO in tissue can induce cell apoptosis [2]. Yu K et al. [3] show that excessive NO in brain tissue can induce cell apoptosis, whereas NO protects endothelial cells from membrane rupture in the physiological manner. Isosorbide mononitrate (ISMN), as an exogenous NO donor, is a commonly used drug in the patients with MS, which is clinically applied for the treatment of cardiovascular disease. However, its exact pathogenesis of apoptosis-promoting effects is unknown. Our study, by observing the apoptosis level of the mice brain with IR and with the treatment of ISMN, explore how ISMN lead to apoptosis in rat brain cells with IR.

2. Materials and methods

2.1 Animals

For this experiment, 14-weeks-old male Wistar rats (weight 393.75±11.09 g) were purchased from Laboratory Animal Center of Henan Province, and the rat weights of each group had no statistical difference.

2.2 Grouping and breeding method

The experiment was randomly divided into 4 groups with 10 rats in each group as follows: Control group (n=10) that received normal food for 12weeks; HFHG group that received High fat & High glucose diet (HFHG) food; ISMN group (n=10) that received

isosorbide mononitrate (4.2mg/kg per d for 12 w,) and normal food for 12 weeks; UNITED group that received High fat & high glucose diet (HFHG) and isosorbide mononitrate. The groups above without the treatment of ISMN received an equivalent volume of saline for 12weeks. The formulation of HFHG was as follow: 79% normal diet, 10% cane sugar, 5% cholesterol and 1% lithocholic acid.

2.3 Sample collection

2.3.1 Blood sample collection

After 12 hours of fasting, rats were narcotized via intraperitoneal injection of 10% chloral hydrate at a dose of 300 mg/kg. 1 milliliter blood was taken by carotid artery intubation. Upper serum was taken by centrifugation and kept in the minus eighty laboratory freezer.

2.3.2 Brain tissue sample collection

After blood was drawn, these rats were killed on the ice workbench, the brains were removed with autopsy, rinsed with ice-cold water. The brain tissue of each rat was cut to 4 pieces (100 mg per piece), one piece was fixed into 4% formaldehyde, and others were put into liquid nitrogen and kept in the minus eighty laboratory freezer.

2.4 Detection index

2.4.1Assessment of apoptosis of rat brain cells

Post-fixed brains were embedded by paraffin, followed by preparation of 6 mm thick using a microtome. The paraffin embedded brain sections were deparaffinized with xylene and rehydrated by ethanol at graded concentrations of 100–70% (v/v), followed by washing with water, and then treated with protease K at 20 ug / ml for 15 min at room temperature. The cells were permeabilized with PBS containing 0.4% Triton X-100 for 15 min. After blocking with 4% BSA, the cells were then incubated in TUNEL kit (Beyotime Biotechnology, Beijing, China) according to the manufacturer's instructions. To assess apoptosis, the numbers of TUNEL stained nuclei were counted by two investigators who worked blindly with regard to the treatments in five randomly selected microscopic fields at × 400 magnification per section.

2.4.2 Blood biochemistry

After overnight fasting, blood samples were taken to assess serum levels of the fasting glucose (FBG, mmol/L) and fasting insulin (FINS, mIU/L) using commercially available kits (Toronto Research Chemicals Inc, TRC). Taking natural logarithm was in order to make HOMA-IR obey normal distribution. Levels of NO and iNOS were measured by Nitrate reduction method.

2.4.3 Molecular biological indicator

2.4.3.1 Real-time PCR for signal mRNA

The expression levels of Bcl-2, Bax mRNA were measured by quantitative real-time reverse transcriptase polymerase chain reaction. Total RNA was extracted from brain tissue with Trizol reagent (TansGen Biotech, Beijing, China) according to the manufacturer's instructions. Total RNA was then reverse-transcribed to cDNA using a cDNA Synthesis kit (TansGen Biotech, Beijing, China) according to the manufacturer's protocol. Real-time PCR was performed with the commercially available kits (TansGen Biotech, Beijing, China). PCR amplification was carried out on the genomic DNA using the following primers: Bax, forward 5'-CACCAAGAAGCTG-

AG-CGAGTG-3'and reverse 5'-ATGTGGGCGTCCCGAAGT-3'; Bcl-2, forward 5'-ATCGCTCTGTGGATGACTG-3' and reverse 5' -TTTGACCATTTGCCTGAAT -3'.

2.4.3.2 Western blot analysis for protein

For western blot analysis, histones were harvested in lysis buffer containing chaps (40mg/ml), tris-hcl (6.305mg/ml), urea (0.4808mg/ml)，PMSF（1mmol/L），aprotinin（5ug/ml），and leupeptin（5ug/ml），and were pulverized in ice water for 3minutes and resolubilized in lysis buffer. Equal amounts of protein (50 ug) for each sample were resolved by SDS-PAGE and transferred to nitrocellulose filter membrane (Millipore, USA). After being blocked for 2h in a buffer containing 5% nonfat milk, the nitrocellulose filter membrane were probed with the following antibodies: anti-bax, anti-bcl2 (1:1,000；Santa Cruz Biotechnology) over-night at 4℃. The membranes were then incubated with Anti-Rabbit antibody produced in goat（Beyotime Biotechnology, Beijing, and China）for 45 minutes at room temperature. Chemiluminescence was detected by an ECL-Plus kit (Beyotime Biotechnology, Beijing, China).

2.5 Statistical analysis

All values are expressed as mean ± SD. The difference in each parameter between 2 groups was compared using two-sample t-test. Comparisons of data among more than 2 groups were performed using one-way analysis of variance (ANOVA) followed by the least significant difference comparison-t test (LSD-t test). A p value < 0.05 was considered statistically significant. All analyses were performed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1 HOMA-IR

HOMA-IR in the groups fed with HFHG and /or ISMN was higher than that of the control group.（$P<0.05$）

Table 1: HOMA-IR

groups	Control	HFHG	ISMN	UNITED
HOMA-IR	2.60±0.28	4.38±0.39a	4.53±0.10a	4.09±0.59a

Data are mean ± SD (n=10). Control, Control group; HFHG, HFHG group; ISMN, ISMN group; UNITED, UNITED group. A $P < 0.05$ for Control vs. other groups.

3.2 Measurement of NO production

The levels of NO in the groups fed with HFHG and/or ISMN were higher than that of the control group. （P<0.05）

The levels of NO in the UNITED group were higher than that of in HFHG group and ISMN group. （P<0.05）

Table 2: Quantitative assessment of NO

groups	Control	HFHG	ISMN	UNITED
NO (μmol/gprot)	5.79±0.66	7.81±0.83b	9.02±0.97b	11.24±1.45 b

Data are mean ± SD (n=10). b $P < 0.05$ for Control vs. other groups.

3.3 Measurement of iNOS production

The levels of iNOS in the groups fed with HFHG and/or ISMN were higher than that of the control group. （P<0.05）

The levels of iNOS in the UNITED group were higher than that of in HFHG group and ISMN group. （P<0.05）

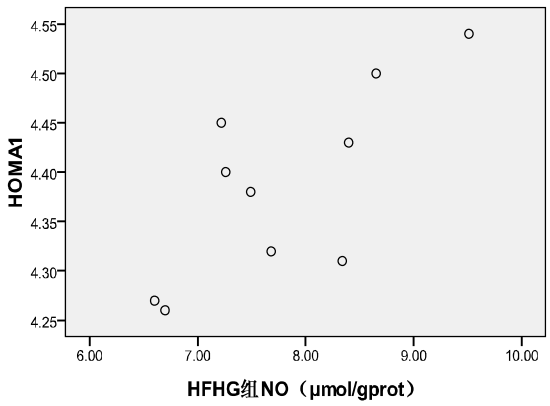
Table 3: Quantitative assessment of iNOS

groups	Control	HFHG	ISMN	UNITED
iNOS (U/mgprot)	10.26±1.31	13.73±1.45 c	16.09±1.77 c	18.75±2.06 c

Data are mean ± SD (n=10). c $P < 0.05$ for Control vs. other groups.

3.4 Correlation analysis between HOMA-IR and NO

The NO level of the rat brain tissue was correlated with HOMA-IR in the HFHG group and the HOMA-IR was significantly correlated with NO level in the ISMN group.



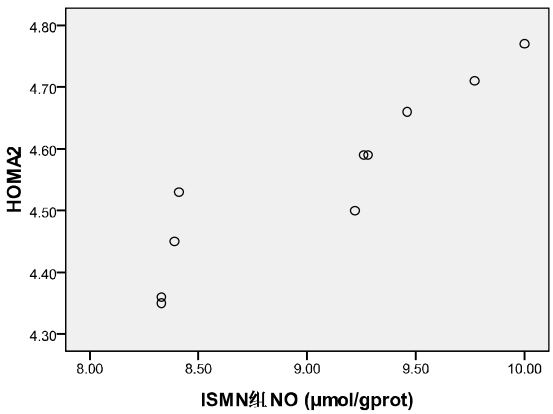


Figure 1: Relationship between NO and HOMA-IR in HFHG and in ISMN

3.5 Assessment of apoptosis of brain cells and apoptotic index

The number of brain cells apoptosis was higher in the groups fed with HFHG and /or ISMN than that of the control group at high magnification（P<0.05）

Table 4: Apoptotic index (AI)

groups	Control	HFHG	ISMN	UNITED
AI (%)	6.33±0.42	9.76±0.57 d	18.37±1.16d	28.65±2.23d

The apoptosis index was expressed as the proportion of the TUNEL-positive brains cells vs. the 1000 total brain cells in percentage.

Data are mean ± SD (n=10). d P < 0.05 for Control vs. other groups.

3.6 Measurement of Bcl-2 mRNA, Bax mRNA and related proteins

Compared with the Control group, the level of Bcl-2 mRNA in HFHG group, ISMN group and UNITED group was decreased. （P<0.05）

Compared with the Control group, the level of Bax mRNA in HFHG group, ISMN group and UNITED group was increased. （P<0.05）

Compared with the Control group, the level of Bcl-2 protein expression in HFHG group, ISMN group and UNITED group was decreased（P<0.05）. Compared with the Control group, the level of Bax protein expression in HFHG group, ISMN group and UNITED group was increased. （P<0.05）

Table 5: Quantitative assessment of Bcl-2 mRNA, Bax mRNA

groups	Control	HFHG	ISMN	UNITED
Bcl-2 mRNA	0.594±0.043	0.486±0.037e	0.304±0.025e	0.186±0.011e
Bax mRNA	0.136±0.009	0.282±0.025 f	0.427±0.031f	0.512±0.043f

Data are mean ± SD (n=10). eP < 0.05, fP < 0.05 for Control vs. other groups.

Table 6: Quantitative assessment of Bcl-2 protein expression, Bax mRNA protein expression

groups	Control	HFHG	ISMN	UNITED
Bcl-2 protein	0.613±0.042	0.491±0.034g	0.317±0.024g	0.139±0.010g
Bax protein	0.084±0.006	0.152±0.011h	0.428±0.026h	0.505±0.042h

Data are mean ± SD (n=10). gP < 0.05, hP < 0.05 for Control vs. other groups.

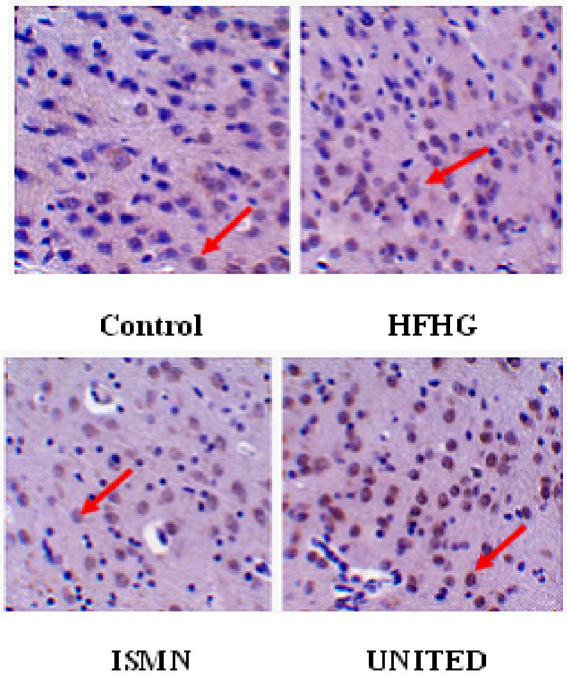


Figure 2: Observed under light microscope (×400), the apoptotic cells were TUNEL positive in light microscopy, and the apoptotic nuclei were stained yellow brown and dark brown, which varied in size and shape from cell to cell; There existed a certain number of apoptotic cells in every group. The mean levels of cell apoptosis in the groups fed with HFHG and / or ISMN were higher than that of the control group.

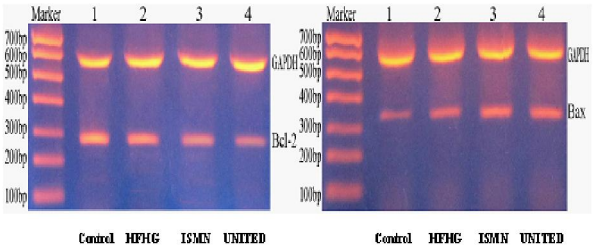


Figure 3: RT-PCR analysis using mRNA samples from the brains of rats in the four groups. The level of Bcl-2 mRNA with the treatment of HFHG and/or ISMN was decreased, whereas the level of Bax mRNA expression increased with the treatment of HFHG and/or ISMN.

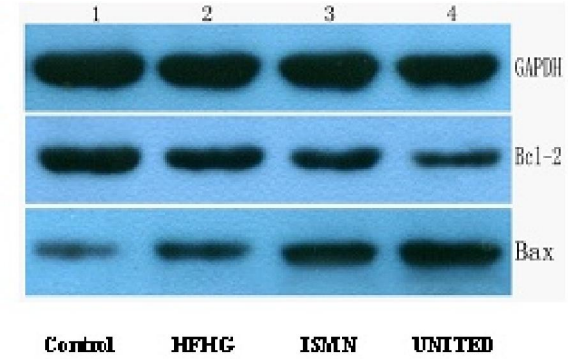


Figure 4: Western blot analysis using protein samples from the brains of rats in the four groups

The level of Bcl-2 protein expression with the treatment of HFHG and/or ISMN was decreased, whereas the level of Bax protein expression increased with the treatment of HFHG and/or ISMN.

4. Discussion

Apoptosis is a highly-regulated, active process of cell death involved in complex apoptosis-inducing molecular mechanisms. Nitric oxide (NO) has becomes a research area of great interest recently, and NO is an important neurotransmitter molecule which acts as either a pro-oxidant or an antioxidant in biological systems [4]. The experiment confirmed that NO can promote apoptosis of rat brain cells which will be more serious when with insulin resistance.

An endothelium derived relaxing factor was discovered by Furchgott [5] and proved its chemical nature is NO by Palmer. Nitric oxide synthases (NOS) catalyze the conversion of L-arginine to NO. NOS has three known isoforms in mammals: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS has a high-affinity binding site for calmodulin and calcium, while iNOS can function in a calcium-independent manner suggesting that any time it is expressed it is likely to produce large amount of active NO [6]. Physiological dose of NO production via increased expression of eNOS regulate vascular tone, protect brain cells and act as a cell-signaling molecule with diverse physiological roles. Numbers of NO produced by iNOS and nNOS have neurotoxic effects [7], and increased peroxynitrite expression via excessive NO binding to O2 or O2- directly block the synthesis of DNA [8], cause imbalance of oxidation-reduction reaction and induce apoptosis. Proapoptotic effects of NO involve a number of related apoptosis-inducing signaling pathways, such as mitochondria-mediated signaling pathway[9], endoplasmic reticulum-mediated signaling pathway[10], Fas-mediated signaling pathway[11] and JNK mediated[12] signaling pathway.

The present study shows that NO and iNOS concentrations in brain tissue in ISMN group were higher than that in Control group and the level of apoptosis were more serious accordingly in ISMN group than that in Control group. These results were consistent with experimental hypothesis. ISMN, as the exogenous NO donor, increases NO level in tissue. NO induces apoptosis if its concentration is enough. The exact molecular mechanism if ISMN might activate iNOS to increase endogenous NO production when providing NO is unknown.

A study shows [13] that NO protects brain cells, but also considerable evidence demonstrates that NO induces apoptosis [14]. In our current study, the reasons of increased levels of apoptosis through the use of ISMN in our experiment are as follows: only male rats were chosen in order to avoid the influence of gender, and the obtained data could not represent all the rats; our study tested the level of NO in brain tissue, while other researches refer to NO in the blood which increases blood flow to the brain tissues; other reports discussed the role of NO in physiological condition, while in our present experiment, male Wistar rats were treated with ISMN （1 mg/kg per d for 12 w），an external NO donor, to investigate whether exogenous NO could induce apoptosis.

Poor glycemic control increases the accumulation of advanced glycation end products (AGEs) [15]. AGEs lead to cellular and vascular wall damage. Previous studies have demonstrated AGEs, by means of interacting with their receptors, enhancing or blunting multiple signaling pathways, contributing to the formation of inflammatory cytokines such as C-reactive protein (CRP), TNF-α （tumor necrosis factor-α） and IL-6(interleukin-6), induce inflammatory response, oxidative stress and cells apoptosis. Taken together, the result of this experiment showed that IR induces apoptosis, which is consistent with the experiment result that the degree of apoptosis was more serious in HFHG group compared to Control group.

In the physiological manner, insulin can increase NO production via activating eNOS activity in endothelial cells [16]. Experimental evidence have suggested that free fatty acid (FFA) decreases NO production and release by down-regulating eNOS expression and activity [17]; Moreover, IR is accompanied with enhanced oxidative stress and NO, combined with the products of large amounts of free oxygen radicals releasing into the blood to generate inactive superoxide, leads to NO inactivation, reduces NO production in vascular cells and contributes to endothelial dysfunction [18], and great levels of nitric oxide might diffuse into tissues via impaired vascular cells.

Evidence demonstrates that insulin at abnormal glucose metabolism increases NO production [19] which might not be induced by eNOS. Increased peroxynitrite expression via NO binding to O2 or O2- directly causes the impairment of DNA and protein. IR might be associated with oxidative stress involved in insulin signaling cascade [20]. Saad M J et al. demonstrated that overexpression of NO induces IR by means of reactive nitrogen species leading to S-nitrosylation of proteins involved in the insulin signal transduction pathway and the inactivation of signal-related kinases [21]. In consequence, excessive NO is involved in the pathogenesis of insulin resistance. In addition, a significant correlation between NO

production and HOMA-IR has been demonstrated in our experiment. In summary, the rolling interaction between NO and IR suggests a promising possible mechanism of insulin resistance which we need to study deeply.

In the present study, we demonstrated that subsequent overproduction of NO in the brain tissue of rat leads to the cell apoptosis, and IR can aggravate the apoptosis; NO and IR work together in the process of the rat brain cell apoptosis through their mutual induction; the effect of NO on promoting apoptosis is associated with NO dose and the difference of inducible enzyme species. More functional and mechanistic studies are warranted to elucidate the possible mechanisms involved, verify the efficacy and safety of ISMN in the prolonged treatment of cardiovascular patients with MS, research new therapeutic targets, and provide a more effective therapeutic approach to the treatment of brain injury and disease via targeting of apoptosis.

References

1. B. Ruiz-Nu´n˜ez, L. Pruimboom, D. A. J. Dijck-Brouwer, and F. A. J. Muskiet, "Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade in animation in an evolutionary context" The Journal of Nutritional Biochemistry, vol.24, no.7, pp.1183-1201, 2013.
2. Opez A, Lorente JA, Steingrub J, et al. Multiple-center, randomized, placebo-controlled, double-blind study of the nitric oxide synthase inhibitor 546C88: effect on survival in patients with septic shock. Crit Care Med, 2004; 32:2le 30.
3. Yu K, Wu Y, Hu Y, et al. Prior exposure to enriched environment reduces nitric oxide synthase after transient MCAO in rats [J]. Neurotoxicology, 2013,39:146- 152.
4. Genovese T, Mazzon E, Paterniti I, et al. Neuroprotective effects of olprinone after cerebral ischemia/reperfusion injury in rats. Neurosci Lett, 2011, 503(2): p. 93-9.
5. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine [J]. Nature, 1980, 288: 373-376.
6. Palmer R M, Rees D D, Ashton D S, et al. L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation [J]. Biochem Biophys Res Commun, 1988,153(3):1251-1256.
7. MEINI A, STICOZZI C, MASSAI L, et al. A nitric oxide / Ca(2+) / calmodulin / ERK1 /2 mitogen-activated protein kinase pathway is involved in the m- itogenic effect of IL-1beta in human astrocytoma cells[J]. Br J Pharmacol, 2008, 153 (8): 1706-17.
8. Choudhari SK, Chaudhary M, Bagde S, et al. Nitric oxide and cancer: a review [J]. World J Surg Oncol, 2013,11(118) 1-11.
9. Zhao Q, Zhang C, Wang X, et al. (S)-ZJM-289, a nitric oxide-releasing derivative of 3-n-butylphthalide, protects against ischemic neuronal injury by attenuating mitochondrial dysfunction and associated cell death [J]. Neuro-chem Int, 2012, 60:134-144.
10. Caldeira MV, Salazar IL, Curcio M, et al. Role of the ubiquitin-proteasome system in brain ischemia: friend or foe [J]? Prog Neurobiol, 2014, 112:50-69.
11. Yin XH, Yan Zhou XY, et al. Neuroprotection of S-nitrosoglutathione against ischemic injury by Down-regulating Fast S-nitrosylation and downstream signaling. [J]. Neurosci, 2013, 248: 290-298.
12. Sui X, Kong N, Ye L, et al. P38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents [J]. Cancer Lett, 2014, 344:174-179.
13. MEINI A, STICOZZI C, MASSAI L, et al. A nitric oxide /Ca 2+) / calmodulin /ERK1 /2 mitogen-activated protein kinase pathway is involved in the mitogenic effect of IL-1beta in human astrocytoma cells [J]. Br J Pharmacol, 2008, 153 (8): 1706-17.
14. Dean DK, Christine T, Nina JS, et al. Neurotoxic nitric oxide rapidly depolarizes and Permeabilizes mitochondria by dynamically opening the mito-chondrial transition pore. Molecular and Cellular Neuroscience.2003, 23: 559-573.
15. Goh S Y, Cooper ME. The role of advanced glycation end products in progression and complications of diabetes. The Journal of Clinical Endocrinology & Metabolism.2008; 93(4):1143-1152. doi: 10.1210/jc.2007-1817.
16. Pansuria M, Xi H, Li L, et al. Insulin resistance, metabolic stress, and atherosclerosis [J]. Front Biosci (Schol ED), 2012, 4: 916-31.
17. MATSUI-HIRAI H, HAYASHI T, YAMAMOTO S, et al. Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence in vitro and in vivo: a relationship between telomeres and nitric oxide [J]. J. Pharmacol. Exp. Ther.2011 Jun; 337 (3):591-9. Epub 2011 Feb 28.
18. TOUSKOVÁ V, HALUZÍK M. Insulin resistance and nitric oxide: molecular mechanisms and pathophysiological associations [J]. Cesk Fysiol. 2011; 60(2):40-7. Review. Czech.

19. Guangda Xiang. Insulin resistance and vascular endothelial function [J]. Chin J Diabetes. 2005, 05: 321-323.
20. STYSKAL J, VAN REMMEN H, RICHARDSON A, et al. Oxidative stress and diabetes: what can we learn about insulin resistance from antioxidant mutant mouse models [J]. Free Radic Biol Med. 2012 Jan 1; 52 (1):46-58. Epub 2011 Oct 20.
21. Saad MJ, Carvalho CR, Thirone AC, and Velloso LA: Insulin induces tyrosine phosphorylation of JAK2 in insulin-sensitive tissues of the intact rat. J Biol Chem 271:22100 –22104, 1996.

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