様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　元 年　 ６ 月　 ５ 日現在

機関番号：１５１０１
研究種目：若手研究(B)
研究期間：2017～2018
課題番号：１７Ｋ１６７９３

研究課題名（和文）Elucidating the mechanism of mitochonria participation in the development of diabetic cystopathy. Is there a role of sirtuin 1 gene?

研究課題名（英文）Elucidating the mechanism of mitochonria participation in the development of diabetic cystopathy. Is there a role of sirtuin 1 gene?

研究代表者

ツナピ　パナイオタ（TSOUNAPI, Panagiota）

鳥取大学・医学部附属病院・プロジェクト研究員

研究者番号：５０７６７４３１

交付決定額（研究期間全体）：（直接経費）　　2,500,000円

研究成果の概要（和文）：2型糖尿病は膀胱の酸化ストレスを有意に誘発した。この現象は膀胱壁、特に尿路上皮における組織変性として確認可能であり、この組織障害が膀胱機能障害につながっていることが示唆された。2型糖尿病による膀胱機能障害は排尿動態検査によって確認できた。さらに2型糖尿病ラットモデルを抗酸化剤で治療にすることにより、膀胱壁の酸化ストレスマーカー発現レベルを有意に低下させ、さらに膀胱の組織学的変化および機能を有意に改善することを解明した。

研究成果の学術的意義や社会的意義
平均寿命はここ150年の間に約2倍まで延長してきているが、この事によりいくつかの問題点も浮上してきている。加齢と強い関連のある糖尿病もその一つであり、健康寿命を延長していく上で、糖尿病の予防法および治療法を確立していくことは喫緊の課題である。本研究の成果は、糖尿病による膀胱機能障害の発症メカニズムを考える上で一つの新たな知見であり、膀胱機能障害の予防法および治療法を確立していく上での基盤になると考えている。

研究成果の概要（英文）：Diabetes mellitus type2 induced significantly increased levels of oxidative stress, both lipid peroxidation and DNA oxidative damage, in the bladder. This had as a result the degeneration and deconstruction of the bladder tissue especially the urothelium. The tissue damage resulted into dysfunction of the tissue as evidenced by the results of the voiding behavior studies. By treating the animals with antioxidants there was a significant decrease in the expression levels of the oxidative stress parameters and subsequently an improvement in the histology and the function of the tissue. Furthermore exercise protocol in combination with the antioxidant treatment had slightly better results than the antioxidant treatments alone, but not statistically significant.
In our study it is of great value that we initiated the antioxidants treatment at an early stage of the diabetes. This resulted into better response of the treatment. Antioxidants may be considered as an effective adjunct therapy.

研究分野：Urology

キーワード：diabetes mellitus　sirtuin 1 gene　sirtuin 1 gene activator　resveratrol　taurine　bladder

様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９、ＣＫ－１９（共通）

１．研究開始当初の背景

Type 2 diabetes has several causes: genetics and lifestyle are the most important ones. A combination of these factors can cause insulin resistance, when the body doesn't use insulin as well as it should. According to the International Diabetes Federation Guideline Development Group (2014), in 2011, there were 336 million diabetic patients around the world (*Diabetes Res Clin Pract* 2014; **103:** 256). Additionally, the same report refers that this number is expected to reach 552 million until 2030. Furthermore, diabetes is one of the leading causes of death in the developed societies. The number of people living with, and dying of diabetes across the world is shocking.

In Japan the number of diabetic patients has been estimated to be 10.1 million. In recent years Japan has been facing demographic changes. According to projections of the population with the current fertility rate, people over 65 years will account for 40% of the population by 2060 and the total population will fall by a third from 128 million in 2010 to 87 million in 2060 (*Ministry of Internal Affairs and Communication, Statistics Bureau. "Japan Statistical Yearbook, Chapter 2: Population and Households"*). Japan's aging rate is currently the highest in the world. Population aging is a major public health concern globally because of the substantial burden that aging-associated diseases place on society. Diabetes mellitus is one of the most common aging-associated diseases affecting the adult population worldwide (*J Diabetes Investig. 2015; 6: 533*). Excess urine is one of these complications which is caused by the diabetes-induced bladder dysfunction or diabetic cystopathy. Diabetic uropathy is found in more than 80% of individuals with diabetes. Bladder cystopathy has been classically described as decreased bladder sensation, poor contractility and increased post-void residual urine diagnosed with urodynamics, uroflow and measurement of post-void residual urine (J Urol. 2009; 182: S8).

Because diabetes induces changes in the urinary tract, a large portion of people who have this disease will develop costly and debilitating urological complications. Therefore we need to expand our knowledge and understanding in this mechanism of diabetes-induced bladder dysfunction in order to develop the best methods of prevention and treatment of the urological complications.

Sirtuins are a highly conserved family of proteins (Trends Cell Biol. 2014; 24:464). They belong to class III histone deacetylase family of enzymes. Sirtuins are implicated in a wide range of cellular processes like aging, transcription, apoptosis, stress resistance and inflammation. Additionally they have a role in energy efficiency and alertness during low-calorie situation (Biochem J. 2007; 404:1).

SIRT1 is a key regulator of metabolism, and its activity is regulated by nutritional status, being up-regulated throughout the body during fasting and calorie restriction. SIRT1 up-regulates mitochondrial biogenesis in several tissues, stimulates fat and cholesterol catabolism in liver, skeletal muscle and adipose tissue, induces the gluconeogenic genes and repress glycolytic genes and activate fatty acid oxidation systemically (Biofactors. 2012; 38:349).

Given their essential function in aerobic metabolism, mitochondria are intuitively of interest in regard to the pathophysiology of diabetes. Type 2 diabetes is associated with both impaired insulin action at target tissues and impaired insulin release.

Defects at both levels are evident early in the course of the disorder, and evidence suggests that mitochondria play a role in both processes. Mitochondria generate energy as electrons are passed from donors at lower to acceptors at higher redox potential through various protein complexes. Along with this process, protons are pumped from the matrix outward, generating a potential difference across the inner membrane. The resulting potential energy is transferred to ATP or dissipated as heat as protons leak back toward the matrix.

Although most electrons are eventually passed to molecular oxygen, a small portion of electrons are leaked during transport. This results in one-electron reduction of oxygen to superoxide, which subsequently is converted to additional radical species. Although the reactive oxygen species generated may be destructive, these radicals also serve metabolic purpose.

In 2010 it has been proved that bladder apoptosis is involved in diabetic cystopathy via activation of the Poly(ADP-ribose) polymerase / c-Jun N-terminal kinase /mitochondrial apoptotic pathway. Therefore we can see that the development of diabetic cystopathy is regulated to some degree by the mitochondrial apoptotic pathway.

２．研究の目的

The purpose of the current research proposal is to elucidate the role of mitochondria in the development of diabetic cystopathy. Furthermore this research proposal aims to investigate the effects of sirtuin-1 activator in the mitochondria function in the bladder in a rat animal model of type 2 diabetes.

３．研究の方法

Six-week-old male Wistar rats were allowed to adjust to the Animal facilities of Tottori University for two weeks. Upon reaching the age of eight weeks, the animals were randomly separated into the Control animals (2 groups +/- exercise protocol) and diabetic animals (8 groups). Diabetes was induced as described: after a 12 h fasting period, the rats followed a combinational procedure which included an intraperitoneal injection of streptozotocin (STZ; 40mg/kg) and high fat diet (HFD). The diabetes was

confirmed by urinary glucose two days after the STZ injection and the diabetic animals were exposed to HFD for eight weeks. Because of the extremely high cost of SRT1720, we used resveratrol, which is also an activator of sirtuin 1 gene. The animals in the treatment groups were as follows: 1. diabetic groups treated with resveratrol at 10mg/kg +/- exercise protocol, diabetic groups treated with resveratrol at 5 mg/kg +/- exercise protocol and diabetic groups treated with taurine at 1g/kg +/- exercise protocol. Furthermore we also had the non-treated diabetic groups +/- exercise protocol that received the vehicle.
The exercise protocol included 5 min training in the treadmill. Treadmill running protocol was performed according to the General Aspects of Animal Care and The Development of Animal Use Protocols. The behavior of each animal was recorded and evaluated. Treadmill was selected because it has a distinct advantage that the total amount of external work done by the rat can be easily assessed.
Three days before the completion of the study the animals were placed in metabolic cages and voiding behavior studies were performed according to methods described by Inoue et al. (BJU Int. 2012; 110: E118). The variables of the micturition reflex obtained were: 1) micturition frequency, 2) total urine output and 3) single voided volume. Subsequently the rats were sacrificed with an overdose of sodium pentobarbital (100 mg/Kg i.p.) Blood was collected from the vena cava; serum was separated, frozen in liquid nitrogen and stored at −80 °C. The bladders were obtained and weighted and cut into small pieces. One piece was processed for histological evaluation, another piece was processed for transmission electron microscopy evaluation and the remaining tissue was frozen at −80 °C and used for the evaluation of molecular parameters.
In order to determine the levels of diabetes-induced oxidative stress levels in the bladder tissue, the concentrations of malondialdehyde (MDA) were measured as a representative and reliable marker for evaluation of lipid peroxidation. A commercially available kit was used (NWLSSTM Malondialdehyde Assay, Northwest Life Science Specialties, LLC, Vancouver, WA, USA), and the method was performed as previously described in our laboratory. The MDA concentrations were normalized by the protein contents. Protein was determined using a commercial kit (Protein Assay Rapid Kit Wako, Wako Pure Chemical, Osaka, Japan).
Immunohistochemistry was performed for lipid peroxidation markers 4-hydroxy- 2- nonenal (4-HNE), MDA and DNA oxidative stress marker 8-hydroxy- 2- deoxyguanosine (8-OHdG). All the samples from each group were stained and evaluated. The samples were deparaffinized in xylene (×3 times×3min), rehydrated in graded alcohols (100%×2 times×3min, 95%×2 times×3min, 70%×1 time×3min, 60%×1 time×3min) and distilled water for 5 min (×1time). Then the sections were subjected to antigen retrieval using a microwave in a 10 mM citrate buffer (pH 6.0) for 10 min (this step was not performed for MDA detection) followed by returning to room temperature by running tap water. For the evaluation of 8-OHdG, the sections were incubated into Triton X-100 for 10 min in room temperature and afterwards the sections were washed with PBS (×3 times). Accordingly, the samples were incubated in 0.3% H2O2 for 15min in room temperature and washing with PBS 3 times for 5 min followed. For saturation of non-specific binding sites, blocking was performed using 1.5% normal horse serum (Vectastain, Peroxidase mouse IgG, PK-4002) for 30 min in a humidified chamber in room temperature. Each diluted first antibody was applied and the samples were incubated overnight at 4°C in a humidified chamber as well. The primary antibodies used were: a) a mouse monoclonal antibody against 4-HNE (1:10, Japan Institute for the Control of Aging, Shizuoka, Japan; Catalog No.: MHN-100P), b) a mouse antibody against MDA (1:50, NOF Corporation, Tokyo, Japan; Product No.: N21 3530), and c) a mouse monoclonal antibody against 8-OHdG (1:15, Japan Institute for the Control of Aging, Shizuoka, Japan; Catalog No.:MOG-100P). The next day, after washing with PBS 3 times for 5 min each, biotinylated horse anti-mouse IgG (1:200) was applied onto the tissue sections and incubated for 30 min in humidified chamber at room temperature. Immunoreaction was performed with an avidin–biotin complex alkaline phosphatase kit (Vectastain, Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with hematoxylin. Negative control sections (i.e., these sections were incubated in the absence of the primary antibody) were also processed and evaluated for specificity or background staining levels.

４．研究成果

Because of high mortality of the non treated diabetic animals we had to make additional experiments in order to have appropriate and equal number of animals within all groups.
In the voiding behavior studies, urine production, micturition frequency, and single voided volume in the diabetic rats (no treatment +/- exercise protocol) were significantly larger than that in the control rats. Resveratrol and taurine treatment slightly improved the voiding behavior but the differences were not statistically significant compared to the diabetic group without treatment. Bladder weights were significantly increased in the diabetic animals compared to the Control animals. Resveratrol treatment significantly decreased the bladder weight compared to the non treated diabetic animals. Histological damages in the tissue of the bladder of the non-treated diabetic groups were prevented in both resveratrol treated groups (+/- exercise).
MDA levels in the bladder were significantly higher in the non-treated diabetic groups compared to the

Control groups and the treatments groups. Additionally the expression of all oxidative stress parameters, as detected by immunohistochemistry was significantly higher in the non-treated diabetic groups compared to the Control or the treatments groups.
At present we are analyzing the data of mitochondrial function parameters in the bladder.

So far our data indicate the significant role of antioxidants treatment in the bladder function as adjunct therapy in the diabetes type-2 animal model which can be enhanced by exercise.

５．主な発表論文等

〔学会発表〕（計 4 件）

1. **P. Tsounapi**, M. Honda, R. Shimizu, R. Nishikawa, S. Teraoka, Y. Kimura, T. Yumioka, N. Yamaguchi, H. Iwamoto, S. Morizane, K. Hikita, A. Takenaka, First stages of diabetes type 2 effects in the bladder; to which extent antioxidant treatment can be beneficial for the bladder tissue? Annual Meeting of the International Continence Society, Philadelphia, Pennsylvania, 2018
2. **P. Tsounapi**, M. Honda, R. Shimizu, R. Nishikawa, S. Teraoka, Y. Kimura, T. Yumioka, N. Yamaguchi, H. Iwamoto, S. Morizane, K.Hikita, N. Sofikitis, A. Takenaka, Changes in oxidative stress parameters in the bladder of a short-period diabetes type-2 rat model, Annual Meeting of the Japanese Urological Association and the Annual Meeting of the Urological Association of Asia, Kyoto, 2018
3. **P. Tsounapi**, M. Honda, R. Shimizu, R. Nishikawa, S. Teraoka, Y. Kimura, T. Yumioka, N. Yamaguchi, H. Iwamoto, S. Morizane, F. Dimitriadis, K. Hikita, N. Sofikitis, A. Takenaka, Can antioxidant therapy be protective against diabetes-induced bladder dysfunction? Annual Meeting of the European Association of Urology (EAU), Copenhagen, 2018
4. **P. Tsounapi**, M. Honda, F. Dimitriadis, K. Hikita, S. Shimizu, M. Saito, N. Sofikitis, A. Takenaka, Oxidative stress-related alterations in the bladder of a short-period diabetes type-2 rat model, Annual Meeting of the American Urological Association, Boston, Massachusetts, 2017.

６．研究組織