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Calcitriol prevents peripheral RSC96 Schwann neural cells from high glucose & methylglyoxal-induced injury through restoration of CBS/H_2S expression

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ABSTRACT

A meta-analysis has suggested that vitamin D deficiency is involved in diabetic peripheral neuropathy (DPN) and the levels of hydrogen sulfide (H_2S) are also decreased in type 2 diabetes. The injection of vitamin D induces cystathionine-β-synthase (CBS) expression and H_2S generation. However, it remains unclear whether the supplementation of vitamin D prevents DPN through improvement of CBS/H_2S expression. In the present study, RSC96 cells, a rat Schwann cell line, were exposed to high glucose and methylglyoxal (HG&MG) to simulate diabetic peripheral nerve injury *in vivo*. Before the exposure to HG&MG, the cells were preconditioned with calcitriol (CCT), an active form of vitamin D, and then CCT-mediated neuroprotection was investigated in respect of cellular viability, superoxide anion (O_2^-) generation, inducible nitric oxide (NO) synthase (iNOS)/NO expression, mitochondrial membrane potential (MMP), as well as CBS expression and activity. It was found that both high glucose and MGO decreased cell viability and co-treatment with the two induced a more serious injury in RSC96 cells. Therefore, the exposure to HG&MG was used in the present study. The exposure to HG&MG markedly induced iNOS expression, NO and O_2^- generation, as well as MMP loss. In addition, the exposure to HG&MG depressed CBS expression and activity in RSC96 cells. However, the preconditioning with CCT significantly antagonized HG&MG-induced cell injury including the decreased viability, iNOS overexpression, NO and O_2^- accumulation, as well as MMP loss. CCT also partially restored the decreased CBS expression and activity triggered by HG&MG, while the inhibition of CBS with hydroxylamine attenuated CCT-mediated neuroprotection. Moreover, the exogenous donation of H_2S produced similar cellular protective effects to CCT. The data indicate that the supplementation of vitamin D prevents HG&MG-induced peripheral nerve injury involving the restoration of endogenous H_2S system, which may provide a basal support for the treatment of DPN with vitamin D clinically.

1. Introduction

Diabetes mellitus (DM), as well as its skin complication, is an increasing public health concern throughout the world, especially in China, where there is the largest diabetic group. The symptoms of diabetic peripheral neuropathy (DPN) include both sensation neuropathy symptoms, such as numbness of extremities and dysesthesia, and autonomic neuropathy symptoms, such as diarrhea, erectile dysfunction and urinary incontinence. However, the decreased sensation is a very early stage of diabetic skin injury and it has been used as an indication for special diabetic footwear. Therefore, it is necessary to discover some medicine or compounds with neuroprotection for the prevention against diabetic skin injury.

Hydrogen sulfide (H_2S) is a well-known gaseous signaling molecule mainly produced by the enzyme cystathionine-β-synthase (CBS) in nervous system and exerts many important

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physiological roles (Dominy and Stipanuk, 2004; Wang, 2012). However, its endogenous generation is severely reduced not only in DM (Jain et al., 2010) but also in neurodegenerative diseases (Eto et al., 2002) including Alzheimer's disease (AD) (Steen et al., 2005). Interestingly, the supplementation of H_2S has been proved to be markedly neuroprotective towards the diseases (Liu et al., 2014; Xuan et al., 2012). As early as 2008, we found that H_2S was able to protect PC12 nerve cells against β-amyloid (Aβ)-induced injury in a cellular AD model (Tang et al., 2008). Consequently, it may be a promising strategy to increase the endogenous H_2S levels by the administration of H_2S synthase agonist or the donation of H_2S for the treatment of DM peripheral nerve injury. Currently there are a great variety of H_2S donors available. Unfortunately, to my knowledge, none of them has been applied in clinical practice yet. Fortunately, many researches have indicated that some ordinary clinical medicine, such as vitamin D, ramipril and carvedilol, appears to boost endogenous H_2S generation (Wilinski et al., 2012). Therefore, we surmise that the supplementation of vitamin D prevents from DM peripheral nerve injury by increasing H_2S generation. Vitamin D is a secosteroid hormone primarily responsible for calcium and phosphate metabolism previously, as well as healthy bone growth. Recently, some evidence has suggested that vitamin D has various other physiological effects. For instance, its deficiency and/or receptor mutation is implicated in many diseases including cardiovascular disease (Ni et al., 2014), neurodegenerative diseases (Millet et al., 2014), as well as insulin resistance and type 2 DM (Pilz et al., 2013), which can be alleviated by the supplementation of vitamin D (Avenell et al., 2009; Ford et al., 2014; Suzuki et al., 2013). A recent meta-analysis indicated that vitamin D deficiency was likely to be associated with DPN in patients (Lv et al., 2014). Therefore, it is interesting to reveal whether vitamin D prevents DM peripheral nerve injury through induction of H_2S generation.

Besides hyperglycemia, the accumulation of advanced glycation endproducts (AGEs) in blood and tissues is a distinct feature in DM patients or model animals (Jack and Wright, 2012). Methylglyoxal (MGO) is an intermediate product in the process of AGEs generation (Jack and Wright, 2012) and derived from Amadori products formed in Maillard reaction between aldehyde group of high glucose and terminal reduced amino of macromolecules. As a very reactive dicarbonyl agent, MGO can crosslink to lots of biological macromolecules by non-enzymatic reaction, produce AGEs and induce a series of toxic effects (Beisswenger et al., 1999; Faure et al., 2005). For example, MGO-modified bovine serum albumin (BSA) can cause insulin resistance and DM status by depleting the endogenous antioxidant defenses (Cai et al., 2012). Some reports indicate that MGO has obvious toxicity to nerve cells through induction of peroxynitrite ($ONOO^-$) stress and mitochondrial dysfunction (Bierhaus et al., 2012; Ray et al., 1994). Nevertheless, it remains unclear whether the excessive MGO/AGEs-induced nervous toxicity can be attenuated by the supplementation of vitamin D. In addition, Schwann cells play an important role in axonal growth, myelin sheath formation and sensory transduction. Under the condition of DM, the dysfunction of Schwann cells causes a defective axonal regeneration and peripheral neuropathy (Tosaki et al., 2008). We accordingly performed experiments to investigate the influence of vitamin D against the high glucose and MGO-induced injury and the mediation of H_2S in rat Schwann cells.

2. Materials and methods

2.1. Materials

Calcitriol (CCT), MGO, glucose, nitroblue tetrazolium chloride (NBT) and hydroxylamine (HA) were bought from Sigma–Aldrich Co. (St. Louis, MO, US). Cell counting kit-8 (CCK-8) and rhodamine123 (Rh123) were purchased from *Dojindo* Laboratory (Kyushu, Japan). The antibody against CBS or iNOS was provided by Bio-world Technology, Inc. (Minnesota, US). Dulbecco's modified Eagle's medium (DMEM) was bought from Gibico BRL (Shanghai, China). Gemcell ™ fetal bovine serum (FBS) was supplied by Gemini company (Woodland, US).

2.2. Cell culture

RSC96 rat Schwann cells (RSC96 cells), a cell line derived from spontaneous transformation of rat peripheral Schwann neural cells, were bought from Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (low glucose) with 10% FBS at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. They were passaged and harvested using 0.25% trypsin/EDTA. After the treatment with CCT, RSC96 cells were cultured in a low FBS medium for 24 h followed by the indicated stimuli.

2.3. Determination of cell viability

The cell viability was measured according to the manufacturer's instruction of CCK-8. RSC96 cells were plated in a 96-well plate at a density of 6, 000 cells/well. When the cells were grown to nearly 70%–80% confluence, the indicated treatments were applied. The CCK-8 solution (100 μL) at a 1:10 dilution with FBS-free DMEM without phenol red was added to each well followed by a further 3 h incubation at 37 °C. The absorbance (*A*) of 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, US). The mean *A* of all wells in each group was used to calculate the percentage of cell viability as follows: percentage of cell viability $= (A_{\text{treatment group}} - A_{\text{blank group}})/(A_{\text{control group}} - A_{\text{blank group}}) \times 100\%$. Experiments were performed for 6 times.

2.4. Western blot assay

After the indicated treatments, RSC96 cells were harvested and split at 4 °C. Total proteins in the cell lysate were quantitated with BCA protein assay kit and fractionated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in gels were transferred into polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free milk (in TBS-T) for 2 h at room temperature and then incubated with the antibody against iNOS (1:3000) or CBS (1:2000) with gentle agitation at 4 °C, and β-actin was used as a loading control. Following three washes with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:4000 dilution) for 1.5 h at room temperature. The immunoreactive signals were visualized using an enhanced chemiluminecence detection system. Image J software was used to quantify the protein expression.

2.5. Measurement of superoxide anion in cells

The intracellular superoxide anion ($O_2^{\cdot-}$) content was measured with NBT, since NBT can be converted to purple formazan by $O_2^{\cdot-}$. Briefly, RSC96 cells were plated in a 96-well plate at a density of 10, 000 cells/well and cultured overnight. At the end of indicated treatments, cell medium was removed and 100 μL NBT solution (1.0 mg/mL in FBS-free DMEM) was added to each well of the plate. After incubation for 2 h at 37 °C, the plate was washed with PBS and then 100 μL stop solution KOH (2 mol/L) and 100 μL DMSO were added. The *A* of 560 nm was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, US). Experiments were repeated at least 6 times.