学 位 論 文 の 要 旨

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学　位　論　文　名	Advanced Glycation End-Products Induce Apoptosis of Vascular Smooth Muscle Cells: A Mechanism for Vascular Calcification
発　表　雑　誌　名	International Journal of Molecular Sciences
（巻，初頁～終頁，年）	(17, 1567, 2016)
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論 文 内 容 の 要 旨

INTRODUCTION

Vascular complication is an important aspect of the pathological course of diabetes mellitus, and affects the disease-related morbidity and mortality. For the development of such complications, hyperglycemia is suggested to play a central role. Hence, in diabetic patients, a long term intensive control of glycemic status is associated with a significantly reduced risk of micro- and macro-vascular complications, which persists even in good glycemic control afterwards. Advanced glycation end-products (AGEs), which are generated by reducing sugars in non-enzymatic reaction with proteins (Maillard reaction), well explain such a “legacy” effect owing to its difficulty in degradation and clearance from the diabetic tissue.

Mönckeberg-type arterial calcification in the media, which is a characteristic feature of patients with diabetes as well as chronic kidney disease (CKD), develops and progresses time dependently. As a mechanism of vascular calcification, vascular smooth muscle cells (VSMCs) apoptosis is reported to be important. Previous studies have reported that AGEs suppressed cellular differentiation and maturation, and induced apoptosis in many types of cells. However, the effects of AGEs on VSMCs apoptosis and its relationship with vascular calcification are largely unknown. Previously, we have demonstrated that glycolaldehyde-derived AGEs induce calcium deposition in rat VSMCs through excessive generation of reactive oxygen species (ROS) and phenotypic transition into osteoblast like cells. In this study, we aimed to investigate the role of glycolaldehyde-derived AGEs on VSMCs apoptosis, with emphasis on the underlying mechanism.

MATERIALS AND METHODS

A7r5 cells (rat aortic VSMC line) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10 % FBS at 37 °C in a fully humidified atmosphere of 5 % CO_2 in air. AGE3-bovine serum albumin (BSA) was prepared by incubating BSA with glycolaldehyde and 5 mM diethylenetriamine pentaacetic acid (DTPA) in 0.2 M phosphate buffer (pH 7.4) at 37 °C for 7 days.

For induction of calcification, growth medium was switched to calcification medium (DMEM containing 10% FBS, 10 mM sodium pyruvate, 10^{-7} M insulin, 100 U/mL penicillin, 100 mg/mL streptomycin, and β-glycerophosphate) after reaching the confluency. The cells were treated with 100 µg/mL of AGE3-BSA or the control BSA (cBSA). Calcium deposition was determined colorimetrically by *O*-cresolphthaleincomplexone method (calcium *C*-test Wako) on day3. Apoptotic cells were identified by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Apoptosis was semi-quantitatively evaluated by the ratio of TUNEL positive cell number divided by Hoechst positive cell number. To quantify apoptosis, an enzyme-linked immunosorbent assay (ELISA) for histone-complexed DNA fragments was also employed. Real-time PCR was performed to determine the mRNA levels for Nox1, Nox4 and $p22^{phox}$ using the QuantiTect SYBR PCR kit. RNA interference technique was used to down-regulate the expression of Nox4 and $p22^{phox}$ in A7r5 cells. SMARTpool small interfering RNA (siRNA) and the reagents for these genes were designed and synthesized by Dharmacon. The expression at the protein level was determined by immunofluorescence and quantified using ImageJ software (http://imagej.net/ImageJ). Dihydroethidium (DHE) assay was performed to determine cellular ROS levels.

Statistical evaluation of the differences between the groups was carried out with unpaired *t*-test and/or one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference.

RESULTS AND DISCUSSION

To examine effects of apoptosis on calcium deposition, A7r5 cells were treated with general caspase inhibitor Z-VAD-FMK (10 µM) or the control Z-FA-FMK (10 µM) for three days. AGE3-BSA-induced calcium deposition was significantly inhibited by the treatment with caspase inhibitor. This suggests that AGE-induced calcium deposition is mediated by apoptotic cell death in VSMCs. Thus, we investigated AGE-induced apoptosis and the mechanism in A7r5 cells.

The cells were treated with cBSA or increasing concentration of AGE3-BSA (25, 50, 100, 200, and 300 µg/mL) with calcification medium. On day 5, apoptotic cell death was measured using an ELISA-based method. Up to 50 µg/mL concentration, AGE3-BSA did not affect A7r5 apoptosis. In contrast, AGE3-BSA significantly increased apoptosis from 100 µg/mL concentration. However, we did not find any dose-dependent effect of AGE3-BSA beyond 100 µg/mL concentration. As AGE3-BSA showed maximum apoptotic effect at 100 µg/mL concentration, we used this dose in all subsequent experiments.

Analysis of apoptosis by TUNEL assay showed that AGE3-BSA markedly increased the number of TUNEL positive cells. Interestingly, pretreatment of cells with NAD(P)H oxidase inhibitor including GKT137831 (20 µM) or VAS2870 (10 µM), markedly decreased the number of TUNEL positive cells. Quantification analysis also showed that the percentage of TUNEL positive cells in a total cell culture population was significantly increased by AGE3-BSA treatment, and such effect of

AGE3-BSA was greatly inhibited by NAD(P)H oxidase inhibitors. These findings suggest that AGE3-BSA-induced apoptosis of VSMC was mediated by the activation of NAD(P)H oxidase.

To evaluate further the roles of NAD(P)H oxidase in AGE3-induced apoptosis of VSMCs, we checked the effects of AGE3-BSA on the mRNA and protein expression of the components of NAD(P)H oxidase. Three days after incubation with AGE3-BSA or cBSA, total RNA was isolated from A7r5 cells, and the mRNA expression was assessed by real-time PCR. The results showed that AGE3-BSA treatment significantly increased the expression of Nox1, Nox4 and $p22^{phox}$ mRNA. These findings were consistent with the protein expression of Nox4 and $p22^{phox}$. Next, we checked whether such increased production of Nox4 and $p22^{phox}$ proteins by AGE3-BSA has any functional significance. In DHE assay, AGE3-BSA significantly increased cellular ROS level compared to medium treated and cBSA treated conditions.

Next, we examined effects of the silencing of Nox4 and $p22^{phox}$ on AGE-induced apoptosis. The real-time PCR results showed that both Nox4 and $p22^{phox}$ mRNA levels were decreased to 5%–20% after mRNA specific siRNA transfection, indicating their sufficient silencing effect. Importantly, AGE3-BSA-induced A7r5 apoptosis was markedly inhibited (42% or 47%) by transfection of either Nox4 or $p22^{phox}$ siRNA, compared to control (scramble siRNA transfection). Double knockdown of Nox4 and $p22^{phox}$ showed a similar inhibitory effect on apoptosis as single gene silencing (42%).

The present study demonstrated that NAD(P)H oxidase-derived oxidative stress is involved in AGEs-induced apoptosis of VSMCs. This might be important to understand the pathogenesis of vascular calcification in diabetes and CKD. Since excessive ROS is thought to stimulate the production of AGEs, the generation of AGEs and ROS might activate a positive feedback loop through NF-κB as well as receptor for AGE (RAGE), leading to the development of cardiovascular diseases.

In vasculature, ROS is generated mainly by NAD(P)H oxidase, which is composed of Nox isoforms, $p22^{phox}$, and associated proteins such as $p47^{phox}$ as a subunit. As previous studies demonstrated that the activity of $p22^{phox}$ as well as Nox4 is associated with their mRNA levels, increased expression of Nox4 or $p22^{phox}$ is most probably responsible for ROS generation. However, we did not find any synergistic or additive effect of double silencing of Nox4 and $p22^{phox}$ on the apoptosis. This result indicates that the presence of both Nox4 and $p22^{phox}$ is essential for functional activity of NAD(P)H oxidase. Moreover, the function of one of them cannot compensate the other components. Since silencing of Nox1 did not affect AGE-induced calcium deposition in our previous study, we speculate that expression level of Nox1 is of little importance for calcium deposition in VSMCs. Future studies need to address the roles of Nox1 and $p47^{phox}$ in VSMCs.

CONCLUSION

AGEs stimulate VSMCs apoptosis through excessive ROS generation. Component of NAD(P)H oxidase such as Nox4 may be a good candidate of new strategy to prevent vascular calcification.