学 位 論 文 の 要 旨

氏名　　多田　裕子

学　位　論　文　名　Advanced Glycation End-Products Induced Vascular Calcification Is Mediated by Oxidative Stress: Functional Roles of NAD(P)H-Oxidase

発　表　雑　誌　名　Hormone and Metabolic Research
（巻: 初頁～終頁等, 年）　(*In Press*)

著　　者　　名　Yuko Tada, Shozo Yano, Toru Yamaguchi, Kyoko Okazaki, Noriko Ogawa, Miwa Morita, Toshitsugu Sugimoto

論 文 内 容 の 要 旨

INTRODUCTION

Vascular calcification, especially medial artery calcification, known as Mönckeberg's calcification is commonly observed in patients with diabetes and end-stage renal disease. Furthermore, calcification of the medial layer of arteries is strongly correlated with cardiovascular complications and future cardiovascular events in type 2 diabetic patients.

Vascular calcification is thought to be regulated process, which is similar to that of bone formation. Although the mechanisms of medial calcification are not fully understood, recent findings suggest that reactive oxidative stress (ROS) in vascular smooth muscle cells (VSMCs) leads to the phenotypic change into osteoblast-like cell and the promotion of calcification process of the extracellular matrix.

Advanced glycation end products (AGEs) accumulated in these patients may be associated with vascular calcification, although their actions are obscure. Some reports show that AGEs promote ROS production, attributing to the development of vascular damage.

Therefore, we investigated an in vitro study to elucidate the effects of AGEs and the roles of NAD(P)H-oxidase in the pathogenesis of vascular calcification.

MATERIALS AND METHODS

We cultured rat A7r5 cells (rat VSMC line) 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.

AGE-BSA were prepared by incubating albumin with 0.1 M DL-glyceraldehyde (AGE2) or glycolaldehyde (AGE3) and 5 mM diethylenetriaminepentaacetic acid in 0.2 M phosphate buffer (pH7.4) at 37 °C for 7 days. For induction of calcification, growth medium was switched to the calcification medium (DMEM containing 10 % FBS, 50 mg/ml ascorbic acid, and β-glycerophosphate) after reaching confluence. The cells were treated with 300μg/ml of control BSA, AGE2, or AGE3. The calcification of the cells was determined using von Kossa staining and the calcium depositon was quantified by calcium C-test (Wako) on day 7. Total RNA was collected on days 3, 5 and 7, and mRNA expressions of osteopontin (OPN), osteocalcin (OCN), Runx2, Nox-1, Nox-4 and $p22^{phox}$ were measured by real-time RT-PCR. After cells were treated with AGE3 for 24 h, the supernatant was collected to determine the concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG), one of the ROS markers. RNA interference technique was used to down-regulate the expression of Nox-1, Nox-4 and $p22^{phox}$ in A7r5 cells.

RESULTS AND DISCUSSION

In rat VSMCs, von Kossa staining showed that both AGE3 and AGE2 markedly stimulated the mineralization. AGE3 seems to be potent to induce calcium deposition similar to or even more than AGE2 in this system. Thus, we performed most of our further experiments using AGE3. Calcium deposition was increased by AGE3 in a dose (100–300 μg/dl) dependent manner. Absolute calcium content was maximum at day 7. However, since AGE3-induced calcium deposition occurred earlier than the control BSA, the ratio was the highest at day 5. Then, we examined whether or not AGEs can induce phenotypic change of VSMC from smooth muscle into osteoblast. Expression levels of OPN, OCN and Runx2 mRNAs were significantly higher in AGE3 treatment than those in control BSA treatment on day 5 or day 7.

In order to see the effects of AGE3-induced oxidative stress in VSMCs, we measured 8-OHdG concentration in the culture medium. After cells were treated with AGE3 for 24 h, 8-OHdG level was significantly elevated in cells treated with AGE3 than those in control BSA, suggesting that AGE3 induces excessive ROS production in VSMCs. Next, we evaluated the effects of AGE3 on mRNA expression of NAD(P)H-oxidase components; Nox-1, Nox-4, and $p22^{phox}$. Increased expression of Nox-1, Nox-4, and $p22^{phox}$ mRNAs (3–6 folds) was observed in cells treated with AGE3. These findings suggest that AGEs promote ROS production at least through activating NAD(P)H-oxidase.

Finally, we examined effects of silencing mRNA for Nox-1, Nox-4, or $p22^{phox}$ on AGE-induced calcium deposition using siRNA transfection method in A7r5 cells. AGE-stimulated calcium deposition was significantly decreased in cells transfected by either Nox-4 or $p22^{phox}$ siRNA. In contrast, there was no significant effect in knockdown of Nox-1. In order to examine cellular phenotype, mRNA expression of osteoblastic marker genes was

measured. Results from real-time PCR showed that AGE3-stimulated up-regulation of Runx2, OPN and OCN was significantly decreased by either Nox-4 or $p22^{phox}$ siRNA transfection.

In the present study, we observed that both AGE2 and AGE3 significantly stimulated calcium deposition in VSMCs. Here, we found that AGE3 increased mRNA expression of Runx2, OPN and OCN, suggesting that AGE-induced calcification might be mediated by the phenotypic change of VSMC into osteoblast like cell, not by just accumulation of calcium ion. AGE3 was more potent in inducing expression of osteoblastic marker genes compared with H_2O_2 whereas less potent in inducing calcification. This discrepancy might be explained by the notion where not only osteoblastic change but also other mechanisms such as apoptosis of VSMC are involved in the calcifying process.

ROS is another known stimulator of the VSMC phenotypic change. We observed that 8-OHdG level in the medium was elevated by AGE3 treatment in VSMCs, indicating that AGEs stimulates ROS production. Previous reports demonstrated that excessive ROS, which is produced mainly by NAD(P)H-oxidase in the vasculature, promotes apoptosis of VSMCs or differentiation into osteoblastic cells. In the preliminary study, we observed that AGE3 stimulated apoptotic cell death in VSMCs. In addition, we found that the mRNA expression of Nox-1, Nox-4 and $p22^{phox}$ was up-regulated by AGE3 treatment and that AGE-induced calcium deposition was significantly suppressed by silencing Nox-4 or $p22^{phox}$, but not by silencing Nox-1. Taken together, these findings indicate that AGEs stimulate osteoblastic phenotypic change and calcium deposition in VSMCs partly through increasing Nox-4 and $p22^{phox}$ mRNA expression followed by ROS production.

CONCLUSION

We found that AGEs stimulate calcium deposition in VSMCs through excessive ROS generation and phenotypic transition into osteoblastic cells. Components of NAD(P)H-oxidase such as Nox-4 and $p22^{phox}$ could be the targets of new strategy to prevent vascular calcification as well as vascular damage.