学 位 論 文 の 要 旨

氏名　　竹野　歩

学 位 論 文 名	Activation of AMP-activated Protein Kinase Protects Against Homocysteine-induced Apoptosis of Osteocytic MLO-Y4 Cells by Regulating the Expressions of NADPH Oxidase 1 (Nox1) and Nox2
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著 者 名	Ayumu Takeno, Ippei Kanazawa, Ken-ichiro Tanaka, Masakazu Notsu, Maki Yokomoto, Toru Yamaguchi, Toshitsugu Sugimoto

論 文 内 容 の 要 旨

INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid formed by the demethylation of methionine and a potent pro-oxidant, and high plasma Hcy levels are often caused by vitamin B12 and folate insufficiency. Accumulating evidence has shown that elevated plasma Hcy level is associated with the risk of osteoporotic fracture. Previous studies have shown that Hcy induces apoptosis of osteoblast lineage cells such as marrow stromal cells and osteoblasts by increasing oxidative stress. However, little is known about the effects of Hcy on osteocytes, which play pivotal roles in bone remodeling.

Oxidative stress is regulated by many oxidant and antioxidant enzymes. Oxidative stress is predominantly induced by NADPH oxidase (Nox), and is prevented by superoxide dismutase (SOD). On the other hand, several studies have shown that AMP-activated protein kinase (AMPK) which plays pivotal roles as an intracellular energy sensor ameliorates oxidative stress by regulating the activities of Nox and SOD in several cell types. However, no study has described the roles of AMPK in osteocytes.

This study aimed to investigate whether Hcy induces apoptosis of osteocytic MLO-Y4 cells through regulating expressions of Nox and SOD, and to determine the effects of AMPK

activation by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and metformin on the Hcy-induced apoptosis of the cells.

MATERIALS AND METHODS

We used MLO-Y4, a murine long bone-derived osteocytic cell line, and the cells were cultured on collagen-coated plates in α-minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in 5% CO_2 at 37 °C. After reaching confluence, the cells were treated with various reagents. Apoptosis was assessed by using DNA fragment detection ELISA assay and TUNEL staining according to the manufacturers' protocol. DNA fragment ELISA assay and TUNEL staining were performed after treatments for 2 days and 3 days, respectively. To investigate the mRNA expression of AMPK subunits (α1, α2, β1, β2, γ1, γ2 and γ3) in MLO-Y4 cells, we performed reverse transcription (RT) PCR. To examine the expressions of Nox and SOD, quantitative real-time PCR and Western blot were performed. For quantitative real-time PCR, total RNA was extracted from the cultured cells using Trizol reagent after 24 hour-treatments to synthesise single-stranded cDNA. We used SYBR green chemistry to determine the mRNA levels of Nox1, Nox2, Nox4, SOD1, and 36B4, a housekeeping gene. 36B4 was used to normalize the differences in the efficiencies of reverse transcription. For Western blot analysis, the cell lysates were collected after 48 hour-treatment and electrophoresed using 10% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were incubated overnight at 4 °C with SOD1 or SOD2 antibody. The blots were then washed, and the signal was visualized using an enhanced chemiluminescence technique.

RESULTS AND DISCUSSION

We first examined the apoptotic effect of Hcy on MLO-Y4 cells. DNA fragment ELISA showed that Hcy treatments (0.1–5.0 mM) induced apoptosis of MLO-Y4 cells in a dose-dependent manner. We confirmed the apoptotic effect of 5 mM Hcy by TUNEL staining. Moreover, to investigate whether oxidative stress is involved in Hcy-induced apoptosis, we examined the effect of N-acetyl-L-cysteine (NAC), an anti-oxidant, on Hcy-induced apoptosis. DNA fragment ELISA showed that the apoptotic effect of Hcy was significantly decreased by co-incubation of 5 mM NAC. Furthermore, we examined whether Nox inhibitors, apocynin and diphenyleneiodonium chloride (DPI), inhibit the Hcy-induced apoptosis of MLO-Y4 cells. DNA fragment ELISA showed that co-incubation of MLO-Y4 cells with 0.1 mM apocynin or 1.0 nM DPI partially but significantly inhibited Hcy-induced apoptosis of the cells. These findings

suggest that oxidative stress and Nox expression underlie the mechanism of Hcy-induced apoptotic effect.

Because no studies have shown that AMPK subunits are expressed in osteocytes, we examined the expressions of AMPK subunits. RT-PCR showed that mRNAs of all subunits were expressed. Then, we examined the effect of AMPK activation by AICAR, an AMPK activator, on the Hcy-induced apoptosis of MLO-Y4 cells. DNA fragment ELISA and TUNEL staining showed that co-incubation of AICAR (0.05–0.1 mM) significantly ameliorated Hcy-induced apoptosis of the cells. We also confirmed that metformin (10–100 μM), another AMPK activator, prevented Hcy-induced apoptosis by using DNA fragment ELISA. Moreover, the favorable effect of metformin was completely cancelled by Ara-A, an AMPK inhibitor. These findings suggest that AMPK activation has protective effects on Hcy-induced apoptosis of osteocytes and that the anti-apoptotic effect of metformin was mediated by AMPK activation.

Then, we investigated the effects of Hcy and AICAR on the expressions of Nox and SOD. Quantitative real-time PCR showed that Hcy significantly increased the expressions of Nox1 and Nox2. Moreover, treatments with AICAR significantly suppressed the Hcy-induced upregulation of Nox1 and Nox2. In contrast, we found that Hcy had no effects on the expressions of Nox4, SOD1 and SOD2. These results indicate that Nox1 and Nox2 may play important roles in Hcy-induced oxidative stress in osteocytes and AMPK activation ameliorates it by regulating the expressions of Nox1 and Nox2.

CONCLUSION

The present study showed that Hcy induced apoptosis of osteocytic MLO-Y4 cells via increasing the expressions of Nox1 and Nox2. Moreover, AMPK activation ameliorated the detrimental effects of Hcy. Therefore, the apoptosis of osteocytes may be involved in the Hcy-induced bone fragility, and AMPK activators such as AICAR and metformin may be useful for preventing the risk of hyperhomocysteinemia-associated fractures.