Simvastatin rescues homocysteine-induced apoptosis of osteocytic MLO-Y4 cells by decreasing the expressions of NADPH oxidase 1 and 2

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Abstract. Clinical studies have shown that hyperhomocysteinemia is associated with bone fragility. Homocysteine (Hcy) induces apoptosis of osteoblastic cell lineage by increasing oxidative stress, which may contribute to Hcy-induced bone fragility. Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, ameliorate oxidative stress by regulating oxidant and anti-oxidant enzymes. However, the effects of statins on Hcy-induced apoptosis of osteocytes are unknown. This study was thus aimed to investigate whether or not statins prevent Hcy-induced apoptosis of osteocytic MLO-Y4 cells and regulate NADPH oxidase (Nox) expression. TUNEL staining showed that 5 mM Hcy induced apoptosis of MLO-Y4 cells, and that co-incubation of 10^{-9} or 10^{-8} M simvastatin significantly suppressed the apoptotic effect. Moreover, we confirmed the beneficial effect of simvastatin against Hcy's apoptotic effect by using a DNA fragment ELISA assay. However, TUNEL staining showed no significant effects of pravastatin, a hydrophilic statin, on the Hcy-induced apoptosis. Real-time PCR showed that Hcy increased the mRNA expressions of Nox1 and Nox2, whereas simvastatin inhibited the stimulation of Nox1 and Nox2 expressions by Hcy. In contrast, neither Hcy nor simvastatin had any effect on Nox4 expression. These findings indicate that simvastatin prevents the detrimental effects of Hcy on the apoptosis of osteocytes by regulating the expressions of Nox1 and Nox2, suggesting that statins may be beneficial for preventing Hcy-induced osteocyte apoptosis and the resulting bone fragility.

Key words: Osteocyte, Simvastatin, Homocysteine, Apoptosis, Statin

STATIN is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which acts as a rate-limiting enzyme for endogenous cholesterol synthesis and is considered a key enzyme in the mevalonate pathway, and it is widely used to prevent cardiovascular events [1-4]. Statin has recently been shown to exert pleiotropic effects on various cells, which may not be directly related to cholesterol synthesis [5]. Since the crucial roles of the mevalonate pathway in bone forming cells such as osteoblasts [6-8] and osteoclasts [9] were reported, the agents inhibiting HMG-CoA reductase have also attracted attention in the field of bone research. Statin induces bone morphogenetic

protein-2 (BMP-2) expression in osteoblasts and stimulates bone formation [10, 11] as well as promotes mineralization [7, 12] and suppresses apoptosis in osteoblastic MC3T3-E1 cells [13].

Previous clinical studies have suggested that elevated plasma homocysteine (Hcy) level is associated with osteoporotic fracture [14-17]. However, the mechanisms underlying Hcy-induced bone fragility have yet to be completely elucidated. We previously examined the effects of Hcy on apoptosis of MLO-Y4 cells, a mouse osteocyte cell line, and showed that Hcy dose-dependently induced the apoptosis of the cells *via* increasing NADPH oxidase (Nox) 1 and Nox2 [18], which are oxidative stress inducing enzymes [19, 20], suggesting that osteocyte apoptosis by oxidative stress may be one of the underlying mechanisms of Hcy-induced bone fragility. On the other hand, many studies have demonstrated that statins ameliorate oxidative stress by regulating Nox expression

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and/or activity in various cell types [21-24], including osteoblasts [25, 26]. Therefore, we hypothesized that statins might prevent Hcy-induced apoptosis. To date, however, there are no reports investigating the effects of statins on osteocytes.

Materials and Methods

Reagents

Cell culture medium and supplements were purchased from GIBCO-BRL (Rockville, MD). Simvastatin, pravastatin and DL-homocysteine were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade commercially available.

Cell cultures

MLO-Y4, a murine long bone-derived osteocytic cell line, was kindly provided by Dr. Lynda F. Bonewald, University of Missouri-Kansas City. Cells were cultured on collagen-coated plates in α -minimum essential medium (α -MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% CO₂ at 37°C. The medium was changed twice a week, and the cells were passaged when they were 80% confluent.

TUNEL staining

Apoptosis of MLO-Y4 cells was assessed by TUNEL staining using an *in situ* cell death detection kit (Roche, Germany) according to the manufacturer's protocol. Briefly, the cells were incubated in chamber slides. After the cells were confluent, agents such as Hcy, simvastatin and pravastatin were added at their specified concentrations and the cells were incubated for 72 h. Fixation, blocking, and permeabilization were then performed according to the manufacturer's protocol. Slides were immersed in TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark. Slides were analyzed using optical microscopy. The average value of TUNEL-positive cells in one microscopic field (200 $\times$) was used to evaluate the degree of apoptosis. TUNEL-positive cells were counted in six or eight randomly selected areas of TUNEL-stained slides, and the average value was calculated.

Apoptosis assay using a DNA fragment detection ELISA kit

Apoptosis was assessed by using Cell Death

Detection ELISA^{PLUS} kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Cells were incubated in 96-well plates, and Hcy and/or simvastatin were added at their specified concentrations after the cells were confluent. After incubation for 60 h, the cells were lysed using 200 μ L of lysis buffer. After centrifugation, 20 μ L of the supernatant was transferred to a streptavidin-coated microplate and exposed to anti-histone antibody (biotin-labeled) and anti-DNA antibody (peroxidase-conjugated) for 2 h at room temperature. Each well was washed 3 times with the incubation buffer, and antibody-nucleosome complexes bound to the microplate were determined spectrophotometrically using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Absorbance measured using the kit was proportional to the degree of apoptosis. Results were expressed relative to the control.

Quantification of gene expression using real-time PCR

We used SYBR green chemistry to determine the mRNA levels of Nox1, Nox2, Nox4 and a house-keeping gene, *36B4*. The primer sequences were: Nox1 forward, 5'-ATGCCCTGCTGCTCGAATA-3' and Nox1 reverse, 5'-AAATTGCCCTCCATTTCCT-3'; Nox2 forward, 5'-ACCGCCATCCACACAATTG-3', Nox2 reverse, 5'-CCGATGTCAGAGAGAGCTATTGAA-3'; Nox4 forward, 5'-CTGCATCTGTCCTGAACCTCAA-3', Nox4 reverse, 5'-TCTCCTGCTAGGGACCTTCTGT-3'; *36B4* forward, 5'-AAGCGCGTCCTGGCATTGTCT-3', *36B4* reverse, 5'-CCGCAGGGGCAGCAGTGGT-3'. Real-time PCR was performed using 1 μ L of cDNA in a 25 μ L reaction volume with ABI PRISM 7000 (Applied Biosystems, Waltham, MA). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the SYBR Green Real-time PCR Master Mix (Toyobo Co. Ltd., Tokyo, Japan) to enable quantitative detection of the PCR product. The PCR conditions were 95°C for 15 min, 40 cycles of denaturation at 94°C for 15 s, and annealing and extension at 60°C for 1 min. *36B4* was used to normalize the differences in the efficiencies of reverse transcription.

Statistics

Results are expressed as means $\pm$ standard error (SE). Statistical evaluations for differences between groups were performed using one-way ANOVA fol-

lowed by Fisher's protected least significant difference. For all statistical tests, a p value < 0.05 was considered statistically significant.

Results

Simvastatin prevented Hcy-induced apoptosis of MLO-Y4 cells

The effects of Hcy and simvastatin on apoptosis of MLO-Y4 cells were examined using TUNEL staining (Fig. 1a and 1b). As previously reported, incubation with 5 mM Hcy significantly increased apoptosis of MLO-Y4 cells ($p < 0.001$) [18]. Co-incubation of 10^{-9} or 10^{-8} M simvastatin significantly suppressed Hcy's apoptotic effect ($p < 0.05$ and $p < 0.001$, respectively), whereas 10^{-8} M simvastatin alone had no effect on apoptosis of the cells. Moreover, we confirmed by using a DNA fragment detection ELISA kit that Hcy-induced apoptosis was significantly inhibited by treatments with 10^{-8} M simvastatin ($p < 0.01$) (Fig. 2).

Next, we examined the effects of pravastatin, a hydrophilic statin, on Hcy-induced apoptosis of MLO-Y4 cells. TUNEL staining showed that pravastatin had a tendency to suppress Hcy-induced apoptosis (Fig. 3a). However, the difference was not significant by quantification of cell count (Fig. 3b).

Simvastatin suppressed upregulation of Hcy-induced Nox1 and Nox2

We examined the effects of simvastatin on Hcy-induced Nox1 and Nox2 mRNA expressions in this study. As shown in Fig. 4a and 4b, Hcy significantly increased mRNA expressions of Nox1 and Nox2 ($p < 0.001$), while co-incubation of 10^{-9} and 10^{-8} M simvastatin significantly inhibited the Hcy-induced upregulation of Nox1 ($p < 0.05$), and 10^{-8} M simvastatin significantly inhibited the upregulation of Nox2 ($p < 0.05$). On the other hand, neither Hcy nor simvastatin affected the expression of Nox4 (Fig. 4c).

Discussion

In this study, we confirmed that Hcy increased the expression of Nox1 and Nox2 as well as induced the apoptosis of osteocytic MLO-Y4 cells as we previously reported [18]. In addition, we showed for the first time that simvastatin ameliorated the Hcy-induced apoptosis and the upregulation of Nox1 and Nox2 in MLO-Y4 cells.

Fig. 1 TUNEL staining of MLO-Y4 cells treated with Hcy and/or simvastatin

TUNEL staining showed that Hcy-induced apoptosis was suppressed by co-incubation of SIM (a). Quantification of cell count of TUNEL-positive cells showed that co-incubation of SIM 10^{-9} and 10^{-8} M significantly and dose-dependently decreased apoptosis of the cells (b). The result was representative of three different experiments. Results are expressed as the mean $\pm$ standard error of mean (SEM). * $p < 0.05$, and *** $p < 0.001$. SIM, simvastatin; Hcy, homocysteine

NADPH oxidases are well-known oxidant enzymes and the dominant source of intracellular oxidative stress. Nox is a complex enzyme composed of two membrane-bound subunits, gp91^{phox} and p22^{phox}, and four cytosolic subunits, p47^{phox}, p67^{phox}, p40^{phox} and small GTPase, Rac1/2 [19, 20]. Statins have been shown to reduce oxidative stress *via* regulating expres-

Fig. 2 Apoptosis assay of MLO-Y4 cells with a DNA fragment detection ELISA

DNA fragment detection ELISA analysis showed that Hcy 5 mM significantly increased the apoptosis of MLO-Y4 cells. Hcy's apoptotic effect was suppressed significantly by co-incubation of SIM 10⁻⁸ M. Simvastatin alone had no effect on apoptosis of the cells. The result was representative of three different experiments. Results are expressed as the mean $\pm$ standard error of mean (SEM). ** $p < 0.01$, and *** $p < 0.001$. SIM, simvastatin; Hcy, homocysteine.

Fig. 3 TUNEL staining of MLO-Y4 cells treated with Hcy and/or pravastatin

TUNEL staining showed that co-incubation of Prava ameliorated Hcy-induced apoptosis (a). Quantification of cell count of TUNEL-positive cells showed that Prava 10⁻⁸ decreased apoptosis of the cells, however, Prava's effect was not significant (b). The result was representative of three different experiments. Results are expressed as the mean $\pm$ standard error of mean (SEM). *** $p < 0.001$. Hcy, homocysteine; Prava, pravastatin

Fig. 4 Effects of Hcy and SIM on expressions of Nox. Real-time PCR showed that 5 mM Hcy significantly increased Nox1 (a) and Nox2 (b) mRNA expressions. Co-incubation of SIM 10⁻⁹ and 10⁻⁸ M significantly decreased the Hcy-induced upregulation of NADPH oxidase 1 (Nox1) (a) and Nox2 (b). The expression of Nox4 mRNA was not affected by Hcy and SIM treatments (c). Results are expressed as the mean $\pm$ standard error of mean (SEM) ($n > 5$). * $p < 0.05$, *** $p < 0.001$. SIM, simvastatin; Hcy, homocysteine

sions and membrane translocations of those Nox subunits in various cells and tissues [21-26]. Huang *et al.* and Yin *et al.* have shown that treatments with H₂O₂ increased Nox4 expressions and induced apoptosis in osteoblastic MC3T3-E1 cells [25] and MG-63 cells [26]. In addition, in their studies, treatment with simvastatin significantly prevented the apoptotic effects of H₂O₂ in the cells [25, 26]. In the present study, we demonstrated the protective effect of simvastatin against Hcy-induced increases in Nox1 and Nox2 expressions and apoptosis of osteocytic cells, suggesting that simvastatin has anti-apoptotic effects on not only osteoblasts but also osteocytes by reducing oxidative stress. In the above studies by Huang *et al.* and Yin *et al.*, simvastatin reduced the expression of Nox4 stimulated by H₂O₂. In contrast, simvastatin ameliorated the expression of Nox1 and Nox2 induced by Hcy in our study. Although the mechanism of induction of oxidative stress by Hcy might be different from H₂O₂, our findings suggest that the dominant Nox subtypes are Nox1 and Nox2, but not Nox4, in osteocytes, and that simvastatin regulates Nox1 and Nox2 expression in the cells.

It is known that the inhibition of the mevalonate pathway induces the differentiation and mineralization of osteoblasts as well as inhibits the activity of osteoclasts and induces osteoclast apoptosis [9]. To our knowledge, this is the first report showing that the inhibition of the mevalonate pathway is important for anti-apoptotic effects in osteocytes. On the other hand, pravastatin did not rescue the Hcy-induced apoptosis of MLO-Y4 cells in this study. There are two types of statin, lipophilic ones, such as simvastatin, and hydrophilic ones, such as pravastatin. Previous studies have shown that lipophilic statins affect osteoblasts and osteoclasts more effectively than hydrophilic ones [6, 27, 28]. Therefore, the effects of lipophilic statins were more potent than those of hydrophilic ones not only in osteoblasts but also osteocytes.

Statins have been shown to exert pleiotropic effects on various cells, which may not be directly associated with cholesterol synthesis. Statins also prevent the synthesis of isoprenoid intermediates such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate as well as small GTP-binding proteins such as Rho, Rac, and Rab, all of which are located downstream of HMG-CoA reductase and have recently attracted widespread attention as the most important factors of

pleiotropic effects by statins [29, 30]. However, the downstream of the inhibition of HMG-CoA reductase in osteocytes is still unknown. Therefore, further studies are needed to determine which downstream molecules are important for the anti-apoptotic effects of statins in osteocytes. On the other hand, we have previously shown that AMPK activation ameliorated Hcy-induced apoptosis of MLO-Y4 cells [18]. Because AMPK is known to inhibit HMG-CoA reductases such as statins [31], the beneficial effects of AMPK might be carried out by the resultant inhibition of the enzyme also in osteocytes. On the other hand, several studies showed that statins activate AMPK in human umbilical vein endothelial cells [32] and neonatal cardiac fibroblasts [33]. Hence, simvastatin might ameliorate Hcy's detrimental effects *via* AMPK activation. Furthermore, statins have been reported to prevent Nox-induced reactive oxygen species (ROS) and apoptosis *via* inhibiting phosphorylation of ERK1/2 [22, 23], p38 [23, 34] and JNK [23] and suppression of the NF- κ B pathway [23] in other cells and tissues. Therefore, further studies are needed to elucidate the underlying mechanism to statins ameliorative effect on oxidative stress in osteocytes.

In conclusion, the present study demonstrated that simvastatin suppressed the upregulation of Hcy-induced Nox1 and Nox2 and ameliorated Hcy's apoptotic effect in MLO-Y4 cells. These results indicate that the inhibition of the mevalonate pathway by simvastatin may have beneficial effects on excess oxidative stress and apoptosis in osteocytes.

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Disclosure

None of the authors has any conflict of interest to declare.

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