HMG-CoA reductase inhibitors up-regulate anti-aging klotho mRNA via RhoA inactivation in IMCD3 cells

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Abstract

Objective: Klotho is thought to play a critical role in the development of age-related disorders including arteriosclerosis. Statins may exert vascular protective effects, independent of the lowering of plasma cholesterol levels. We investigated the impact of statins on mRNA expression of the age-suppressor gene, klotho in mIMCD3 cells.

Methods and results: Klotho mRNA levels were evaluated with real-time RT-PCR. Atorvastatin and pitavastatin increased the expression of klotho mRNA in a dose-dependent manner. This stimulatory effect was abolished by the addition of mevalonate, GGPP and FPP, essential molecules for isoprenylation of the small GTPase Rho. As was the case with the statin treatment, inhibition of Rho-kinase by Y27632 up-regulated klotho mRNA. In contrast to the statin treatment, stimulation with angiotensin II down-regulated klotho mRNA expression without obvious morphological changes. Furthermore, pretreatment with atorvastatin blunted the angiotensin II-induced response and ameliorated the decrease in klotho mRNA expression towards basal levels. RhoA activity was further evaluated by detection of its translocation. Angiotensin II activated RhoA, whereas statins potently inactivated RhoA and blocked RhoA activation by angiotensin II.

Conclusion: Statins inactivate the RhoA pathway, resulting in over-expression of klotho mRNA, which may contribute to the novel pleiotropic effects of statins towards vascular protection.

Keywords: HMG-CoA reductase inhibitors; Klotho; RhoA; Real-time RT-PCR

1. Introduction

The 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, known as statins, are effective in lowering plasma concentration of LDL-cholesterol and are widely used in patients with hypercholesterolemia [1,2]. Recently, statins have been shown to decrease the incidence of myocardial infarction and other ischemic vascular events independent of their lipid lowering properties [3,4]. Furthermore, experimental and clinical evidence indicates that the “pleiotropic” effects of statins involve improving or restoring endothelial function, enhancing the stability of atherosclerotic plaques, and decreasing oxidative stress and vascular inflammation [5–7]. By inhibiting L-mevalonic acid synthesis, statins prevent the synthesis of other isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesyl pyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) [1]. These intermediates are important lipid moieties for the posttranslational modification of proteins, including the γ subunit of heterotrimeric G proteins, Heme-a, nuclear lamins, and small GTPases of the Rho and Ras family [8,9]. Because Rho is a major target of geranylgeranylation, inhibition of Rho and its downstream target, Rho kinase, is a likely
mechanism to mediate some of the pleiotropic effects of statins [10], and inhibition of Rho isoprenylation mediates many of the cholesterol-independent effects of statins in various cells [11,12].

The klotho gene, identified by insertional mutagenesis in mice, is a suppressor of the expression of multiple aging phenotypes similar to age-related disease, such as arteriosclerosis, infertility, skin atrophy, osteoporosis and pulmonary emphysema [13]. Klotho mRNA and protein are predominantly expressed in the kidney. Recently, several studies have shown that klotho gene delivery improves endothelial dysfunction and is involved in modulating vascular function (i.e., hypertension, vascular remodeling) [14] and ameliorates renal damage induced by angiotensin II (AngII) [15]. Remarkably, in humans, the klotho allele is associated with early-onset occult coronary artery disease [16] and longevity [17]. These reports suggest that klotho allele expression contributes to heterogeneity in the onset and severity of age-related phenotypes in humans [17].

We hypothesized that the klotho gene has characteristic roles on pleiotropic effects of statin. We have recently shown that statins enhance the expression of klotho protein, which was reduced in hypertension and vascular injured rat models induced by chronic administration of L-NAME and AngII (manuscript in press). To date, however, no report is available on the expression of the klotho gene in established cultured cell lines, and the regulatory mechanism(s) of klotho gene expression remains poorly understood. The purpose of this study was to analyze the impact of statins on klotho gene expression in vitro. Klotho expression is detected especially in renal tubular epithelial cells, so we use the established tubular cell line, IMCD3 cells, in this study. The characters of IMCD3 cells were similar to the renal tubular cells in vivo from the viewpoint of klotho expression and morphology. To further elucidate the underlying mechanism(s) in the modulation of klotho expression by statins, we investigated the role of isoprenylation of the small GTPase, RhoA.

2. Material and methods

2.1. Reagents

AngII was purchased from Peptide (Osaka, Japan). Mevalonate, farnesy pyrophosphate (FPP) and geranylgeranylporphosphate (GGPP) were purchased from Sigma (St. Louis, MO, USA). Y-27632 (4-pyridyl)-(4-aminoethyl)-cyclohexane carbox-amide) was purchased from Chemicon (Temecula, CA, USA). Mevalonate was activated chemically with alkaline hydrolysis. Atorvastatin was kindly provided by Pfizer (New York, NY, USA). Pitavastatin was a generous gift from Kowa Shinyaku (Tokyo, Japan). Mouse anti-RhoA monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other materials used were commercial products of the highest grade available.

2.2. Cell culture

Mouse internal medulla collecting duct epithelial cells (IMCD3) were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium/HAM’s F12 (Gibco-BRL, Rockville, MD, USA) and supplemented with 10% FBS and 100 U/ml of penicillin/streptomycin in a humidified 5% CO₂ incubator, maintained at 37 °C. Cells at 80%—full confluence in 60-mm culture dishes were made quiescent through incubation in medium with 1% FBS for 24 h prior to the experiment.

2.3. Immunocytochemistry

For microscopic examination, cells were cultured in 4-well glass chamber slides (Nunc, Gibco/BRL life Technologies, Roskilde, Denmark). Following stimulation under indicated conditions, the cells were washed with sterile PBS and fixed in 3% paraformaldehyde for 15 min at room temperature. Slides were blocked with 10% goat serum/PBS for 60 min prior to a further washing step with PBS. For staining of E-cadherin, cells were visualized by fluorescence microscopy and representative pictures were documented, using DP70 image analysing software (Olympus, Tokyo, Japan). Photographic images were taken from five random fields by a photographer who was unaware of cell origin.

2.4. Quantification of RNA

In all experiments following isolation of total cellular RNA, klotho and β-actin mRNA levels were examined by real-time reverse transcription (RT) and polymerase chain reaction (PCR) by LightCycler (Roche Diagnostics, Basel, Switzerland). Total RNA samples from the cultured IMCD3 cells were isolated using RNeasy RNA extraction kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer’s protocol. First-strand cDNA was synthesized with 2 μg of total RNA as a template using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with 20 μl of LightCycler DNA-Master, SYBR Green I, 25 pmol of oligonucleotide primers, 2 μl of cDNA solution and 2.4 μl of 25 mmol/l MgCl₂. After initial denaturation at 95 °C for 10 min, reactions were cycled 40 times using following parameters for klotho detection: 95 °C for 5 s, primer annealing at 58 °C for 10 s, and primer extension at 72 °C for 20 s. Beta-actin cDNA was amplified as follows: 95 °C for 5 s, primer annealing at 58 °C for 10 s, primer extension at 72 °C for 20 s.
Specific oligonucleotide primer sequences were as follows. For mouse klotho, forward: 5’-ctggttgcccacaacctact-3’; reverse: 5’-tcgagagagagagagaaaa-3’ (amplifies a fragment of 321 bp). For mouse β-actin, forward: 5’-tgagctgagagagagaca-3’; reverse: 5’-tcgagagagagagagagaaaa-3’ (amplifies a fragment of 387 bp). SYBR Green I fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during the cycle. At the end of each run, melting curve profiles were produced to confirm amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and quantified using the second derivative maximum method with the LightCycler Software (Roche Molecular Biochemicals). Standard curves for klotho and β-actin were constructed using serial dilutions of total cDNA from Balb/c mouse kidney tissue. The standard curve samples were included in each run. Standards for both klotho and β-actin were defined to contain an arbitrary starting concentration, since no primary calibrators exist. Hence, all calculated concentrations are relative to the concentration of the standard.

2.5. Western blot analysis

The quiescent IMCD3 cells were washed with PBS and microcentrifuged for 5 min at 1500×g. For preparation of whole-cell lysates, the pellet was resuspended in 500 µl of lysis buffer (10 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 25 mmol/l EDTA, 5 mmol/l EGTA, 0.25% sodium deoxycholate, 1 mmol/l DTT, 0.1 mg/ml PMSF, 2 mg/ml leupeptin). For preparation of cellular membranes, membranes were fractionated and purified, as described previously [18,19]. Equal amounts of protein (20 µg) from each fraction were subjected to SDS-PAGE analysis, followed by immunoblotting with anti-RhoA antibody. The immunoreactive RhoA proteins were visualized using enhanced chemiluminescence (Amercham Bioscience Piscataway, NJ, USA), according to the manufacturer’s instructions.

2.6. Statistical analysis

Data are presented as the means±S.E.M. from at least six independent experiments. Statistical analyses were performed with ANOVA followed by Fisher’s protected least significant difference test for multiple comparisons. A level of p<0.05 was considered statistically significant.

3. Results

3.1. Cell culture

We characterized the cells by their morphological features and the presence of E-cadherin, an epithelial cell-specific protein. The concentration of atorvastatin in clinical usage is thought to be about 0.025 to 0.185 µmol/l, but to clarify the adverse effects of statins on IMCD3 cells, we chose the concentration of atorvastatin in this experiment at 1 µmol/l. Adverse effects on the characteristics of the IMCD3 cells by atorvastatin (1 µmol/l) were not evident following 24-h exposure (Fig. 1B,G). Stimulation with AngII (10 nmol/l) did not evoke morphological changes within 12 h (Fig. 1C,H), but after 24-h stimulation, cells began to separate and to acquire a spindle-shaped morphology and the expression of E-cadherin on the membrane was lost (Fig. 1D,I). Pre-incubation with atorvastatin prevented these changes induced by AngII stimulation (Fig. 1E,J).
3.2. HMG-CoA reductase inhibitors up-regulate klotho mRNA expression in IMCD3 cells

To examine whether treatment with statins up-regulates klotho expression in vitro, IMCD3 cells were cultured for 24 h in the presence of increasing concentrations of atorvastatin (Fig. 2A) and pitavastatin (Fig. 2B). Stimulation with atorvastatin (0.01, 0.1 and 1 μmol/l) dose-dependently up-regulated the klotho mRNA by 1.8-, 3.8- and 3.7-fold, respectively (n=6 to 8; p<0.05 versus control). Stimulation with pitavastatin (0.1, 1 and 5 μmol/l) also up-regulated the klotho mRNA by 1.2-, 1.5- and 3.5-fold (n=6 to 8; p<0.05 versus control), suggesting a class effect, rather than a drug-specific effect, of the statins, accordingly the following experiments were performed using only atorvastatin. A previous study has demonstrated that AngII down-regulates renal klotho mRNA expression in vivo [16]. Hence, to investigate whether AngII would decrease klotho expression and whether statins would ameliorate the down-regulated klotho expression by AngII in vitro, IMCD3 cells were cultured with AngII in the presence or absence of atorvastatin (Fig. 3). Stimulation with 10 nmol/l AngII suppressed the expression of klotho mRNA in time-dependent manner. Furthermore, pretreatment with atorvastatin prevented the inhibition of klotho mRNA expression by AngII, rather increased over their basal level.

3.3. Isoprenylation role of Rho kinase on expression of klotho mRNA

To determine the role of RhoA geranylgeranylation in regulating the expression of klotho mRNA, cells were treated with several intermediates, including FPP, GGPP and mevalonate. We referred to the previous study [19–21] in fixing the concentrations of these materials. As shown in Fig. 4, mevalonate completely abrogated the up-regulating effect of atorvastatin on klotho mRNA expression. Likewise, FPP and GGPP also prevented the up-regulation of klotho expression by atorvastatin. In addition, to examine whether the Rho/Rho kinase system mediated the effect of atorvastatin on klotho mRNA expression, IMCD3 cells were cultured with Y-27632, a pharmacological inhibitor of Rho kinase [22,23]. Treatment with Y-27632 (10 μmol/l) increased the expression level of klotho mRNA by 2.4 fold (Fig. 4), suggesting that Rho/Rho kinase is critically involved in the regulation of klotho mRNA expression.
3.4. Effects of statins on the membrane localization of RhoA

Because membrane translocation of RhoA from the cytosol is necessary for their proper function, we studied the effect of atorvastatin on translocation of RhoA in IMCD3 cells. A direct effect of atorvastatin on the modification of RhoA was demonstrated by Western blot analysis of membrane-bound and total cellular RhoA protein (Fig. 5). Atorvastatin treatment (0.1 μmol/l, 24 h) significantly decreased the amount of membrane-bound RhoA proteins compared with control. Stimulation with AngII (10 nmol/l, 6 h) increased the amount of membrane-bound RhoA, but pretreatment with atorvastatin restored the distribution of cytosol- and membrane-bound RhoA proteins induced by AngII. The levels of total cellular RhoA protein were not significantly different among each sample.

4. Discussion

In the present study, we demonstrated three major findings. First, klotho mRNA was detected in established cultured cell lines by real-time RT-PCR for the first time to our knowledge. Secondly, statins dose-dependently up-regulated klotho mRNA expression via suppression of the small GTPase, RhoA. Finally, without exhibiting tubular epithelial cell damage, AngII reduced klotho mRNA expression, which was ameliorated by pretreatment with statin. These findings suggest that inactivation of RhoA, initiated by the statins, is essential to the up-regulation of klotho expression.

At present, the mechanism(s) regulating the klotho gene is poorly understood. In the view of modulation of klotho gene, only one report has demonstrated that iron inhibited renal expression of klotho at both the mRNA and the protein levels in vivo [24]. One of the reasons for this lack of evidence is that the klotho gene has not been detected in established cell lines to date. We demonstrated the expression of klotho mRNA in IMCD3 cell line, allowing easier and more rapid investigation of the klotho gene function. Klotho is closely related to the regulation of calcium homeostasis [25] and the deterioration of calcium homeostasis is the major cause of aging related disorders including arteriosclerosis and ectopic calcification in artery in klotho mutant mice [13]. On the other hand, calcium homeostasis is maintained in renal tubular epithelial cells and statins may affect bone formation [12]. These results suggest that klotho expression, renal tubular epithelial cells, and calcium metabolism are closely related each other in vivo, and to clarify the interaction between these factors will be an important subject of future studies. Curiously, two different types of klotho protein, namely the membrane-associated and the secreted protein, have been identified as a result of alternative RNA splicing [13]. Although klotho expression is detectable only in kidney and brain, the klotho knock out mouse shows severe changes in many organs. In addition, aortic relaxation in response to acetylcholine improved upon parabiosis of wild-type mice and mice heterozygous for deficiency of the klotho gene [26]. Regrettably, RT-PCR quantitative analysis of each form of klotho mRNA was not successful. At the protein level, we could detect klotho protein in cells only after statin stimulation.

Enhancement of klotho mRNA expression by statins apparently result from the inhibition of HMG-CoA reductase. Products of mevalonate pathway, the isoprenoid lipids FPP and GGPP, are critically involved in the regulation of the activity of the Rho and Ras family [8]. RhoA been shown to translocate from the soluble to the particulate fraction upon activation [27] and as described previously [28], statins did not increase the pool of RhoA available for signaling as shown in Fig. 5. A specific inhibitor of RhoA, Y-27632, had similar effects to statins on klotho mRNA expression. Taken together, these results suggest that the inactivation of Rho/Rho-kinase is involved in the induction of klotho mRNA expression by statins. Atorvastatin seems to be more effective on klotho mRNA expression than pitavastatin. Although the underlying mechanism remains undetermined, it is possible that atorvastatin inhibits Rho/Rho kinase more potently than pitavastatin.

AngII plays an important role in the development of renal injury and fibrosis. New research suggests that myofibroblasts may derive from the tubular epithelium via epithelial mesenchymal transition (EMT) [29]. Numerous signaling pathways have been described as mediators of EMT, and AngII has also been reported to induce this process [30]. Cells induced towards the EMT pathway exhibit a myofibroblast phenotype with loss of epithelial cell characteristics, and loss of E-cadherin protein [31]. A previous report has demonstrated that continuous administration of AngII decreases klotho mRNA expression in the rat kidney [16]. However, renal tubular epithelial cells underwent atrophic changes and resulted in EMT following AngII stimulation. We surmised that these renal tubular cells might not express klotho mRNA. Our data demonstrated that AngII directly down-regulated klotho mRNA with activation of RhoA, and pretreatment with statins attenuated the effects of AngII on klotho mRNA expression. Based on the data presented in Fig. 5, the mechanism of this effect of

![Fig. 5. Effects of atorvastatin and angiotensin II on RhoA translocation. Cells were stimulated with angiotensin II (Ang II; 10 μmol/l) for 6 h in the presence or absence of atorvastatin (Ator; 0.1 μmol/l). Total cell lysates and membrane fractions were analysed by Western blotting. Blots are representative of the results of three separate experiments.](image-url)
statins could be direct inhibition of translocation of RhoA. Additional pathways, including the down-regulation of AngII type 1 receptor expression, which exist in mouse collecting duct epithelial cells [32], or up-regulation of nitric oxide synthase, cannot be ruled out. However, these events were previously described in smooth muscle cells [33], and endothelial cells [7,34], but not in epithelial cells. Alternatively, the involvement other mechanism such as Akt activation or PI3K pathway can not be ruled out, therefore more detailed studies are expected in the future.

In conclusion, this study provides new and important information on the functional significance of statins in the modulation of klotho mRNA expression through inhibition of RhoA in established cell lines. Although, at present, little is known about the mechanisms of action of the klotho proteins, novel vascular protective effect of statins by up-regulating anti-aging klotho expression via Rho/Rho-kinase inhibition was suggested.

References